Interactions between the peach aphid Myzus persicae and the entomopathogenic fungus Verticillium lecanii

Roditakis, Emmanouil

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Interactions between the peach aphid *Myzus persicae* and the entomopathogenic fungus *Verticillium lecanii*

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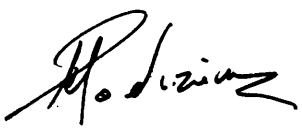

E. Roditakis

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

'Those who cannot remember the past are doomed to repeat it'

George Santayana

CONTENTS

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Abbreviations

200x	Times of magnification under microscope
ANOVA	Analysis of variance (one way)
BPA	Bacteria peptone agar media
°C	Degrees Celsius
cfu	Colony forming units
cm	Centimetre
cryo. SEM	Cryo scanning electron microscopy
d.f.	Degrees of freedom
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
EBF	(*E*)-*β*-farnesene
EPG	Electrical penetration graph
gr	Gram
h	Hour
kb	Kilobase
kdr	Knockdown resistance
L : D	Light : dark (photoperiod cycle)
LC_{50}	Median lethal dose: dose taken to kill 50% of organisms in a population
LD_{50}	Median lethal concentration: concentration taken to kill 50% of organisms in a population
m	Meter

ml	Millilitre
mm	Millimetre
n	Number of sampling units in a sample
P	Probability
π	Pi
PC	Personal computer
PCR	Polymerase chain reaction
ppm	Parts per million
r	Product moment correlation coefficient of a sample
r^2	Coefficient of determination
RAPD	Random amplification of polymorphic DNA
rpm	Rounds per minute
SD	Standard deviation
s.e.	Standard error
sec	Second
SEM	Standard error of the mean
sp.	Spores
UV	Ultra violet light
w/v	Weight/volume
X^2	Chi-square statistic test

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Abstract

The aphid *Myzus persicae* is a major crop pest, that has developed resistance to major groups of insecticides over the past few years. The present work was aimed at improving the control achieved by an alternative pest control method, the use of entomogenous fungus *Verticillium lecanii*.

Four *M. persicae* clones, with different resistance levels, were studied; S (susceptible clone), R1 (low resistant), R2 (medium resistant) and R3 (highly resistant). No differences were found between the dose responses of the four clones to *V. lecanii* (Vertalec) when the fungal inoculum was applied *in situ*. The dose response of the two extreme clones, R1 an R3, was also studied using two more spore application methods. The 'direct impact' method, was designed to allow spore pick up only from the applied spore suspension, and consequently known levels of inoculum. Absence of significant differences using this application method suggested absence of susceptibility differences to the fungus. The 'secondary pick up' method allowed spore pick up only from previously inoculated leaf surfaces, and as a result of the above findings, the absence of dose response differences was associated with possible absence of differences in activity between insects of the two clones.

Visual analysis of time-lapse video recordings of aphid behaviour, suggested that both susceptible and resistant aphid clones (S and R3) demonstrated similar and very low activity levels during the experiments The two clones also exhibited similar honeydew excretion rates, closely associated with feeding rates. Similar low activity levels were recorded for clone R1 using computer analysis (tracking program) of aphid mobility. Differences were found in the reproductive rate between the aphid clones but there was no correlation with the level of resistance.

Visual analysis of behaviour of *V. lecanii* infected aphids indicated possible increase of aphid activity in the first days of the infection development. Indeed, computer

analysis indicated that an aphid infected with isolate Vertalec covered significantly more distance that control aphids 1 and 2 days after application. Using the isolate KV42 the aphids demonstrated increased mobility only one day after application. This was associated with differences in host attack strategies between the two isolates.

Aphid alarm pheromones (EBF) increased the activity of the aphid clones, resulting in significantly higher mortality due to enhanced secondary spore pick up. However, EBF occasionally produced inconsistent results due to its unstable chemical structure. Use of sublethal systemic doses of the insecticide imidacloprid also significantly increased aphid mobility and mortality in secondary pick up experiments. There was no synergism between the fungus and the insecticide other than increased levels of spore pick up. Use of sublethal doses of imidacloprid, as an irritant and antifeedant, could possibly provide a practical method to improve fungal efficiency in field applications.

CHAPTER ONE

General Introduction

1.1 Aphid *Myzus persicae* as a pest

Aphids evolved 280 million years ago and their most striking features, small size and their ability to reproduce both sexually and asexually can still be found in the modern aphids (Dixon, 1998).

Myzus persicae (Sulzer) (Hemiptera: Aphidadae) (common name: green peach aphid or peach potato aphid) probably came initially from Asia but now has a world-wide distribution (Figure 1.1). *M. persicae* became a major pest of many crops mainly because it is an efficient vector of plant viruses (McKinlay *et al.*, 1992).

1.1.1 Description and life cycle

Aphid life cycle is characterised by a sequence of morphs. This characteristic of aphids is called polyphenism. Morphs differ in behaviour, physiology and structure. Morphs also differ in their allocation of resources to reproduction (parthenogenetic

apterae), to dispersal (alatae) or to defence (aestivation or soldiers, morphs absent for *M. persicae*) (Dixon, 1998).

The adult parthenogenetic apterae is small to medium sized (length: 1.25-2.50mm) with a soft barrel-shaped body (Figure 1.2). Its colour can be green to yellowish with a dark thorax and a dark mark on the abdomen. The antenna is 2/3 of the body length. The siphunculi are slightly swollen with dark tips (Hill, 1987). Adult alatae are of similar size to the apterae. The colour is similar or darker with a central black patch on the upper surface of the abdomen. Their reproduction capacity is restricted compared to apterae (Dixon, 1998; Hill, 1987; McKinlay *et al.*, 1992).

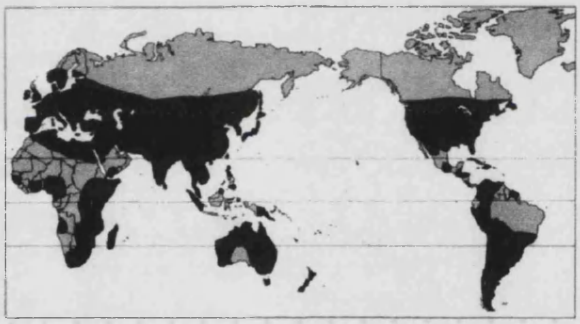


Figure 1.1 Distribution of *M. persicae* in the world (from Hill, 1987)

Figure 1.2 Adult *Myzus persicae* (Sulz.)

The life cycle of aphids can be either holocyclic or anholocyclic. Holocyclic life cycles have an annual sexual phase whereas anholocyclic life cycles do not produce sexual forms. There is yet another form of reproduction, in *M. persicae* and some other aphid species, described as androcyclic, in which only sexual males and viviparous females are produced (Dixon, 1998).

The holocyclic life cycle starts from a 'winter egg' laid on the bark of a peach tree (primary host) by the oviparae, sexual female aphids. From the 'winter egg' a parthenogenetic female hatches, this is called the fundatrice. These aphids will give birth to the fundatrigeniae, also parthenogenetic. Eight generations may pass on peach trees before the winged (alatae) forms are produced and fly to the secondary hosts. Aphids like *M. persicae* that alternate annually between primary and secondary hosts are called heteroecious. Secondary hosts are found among more that 40 different plant families (e.g. Chenopodiaceae, Leguminosae, Solanaceae, Compositae, Cruciferae, Cucurbitaceae). On these plants the alatae give birth to parthenogenetic apterae. At the end of the summer, sexual alatae forms (males and females) triggered by the declining day length and temperature, will appear. The sexual alatae return to the primary host where the female alatae (gynoparae) give birth parthenogenetically to sexual females (oviparae). The oviparae after mating, will subsequently lay winter eggs. Thus, the cycle is completed (Dixon, 1998; McKinlay *et al.*, 1992).

In UK *M. persicae* reproduces sexually only in areas that the primary host (peach tree) is present. In general, *M. persicae* overwinters as a viviparous adult (alatae) (Devonshire *et al.*, 1998; Foster *et al.*, 1996).

1.1.2 Host damage

M. persicae is a polyphagous sap sucking insect. Aphids in general, feed on the phloem by penetrating the plant tissue with their stylets (Dixon, 1998). Parthenogenetic apterae have a very high reproductive rate and can build up large numbers very fast; this is partially the reason for their pest status (Dixon, 1987). *M. persicae* can directly damage the infested host by competing for nutrients and by causing water stress, resulting in reduced final yields. The aphid saliva is secreted during tissue penetration and feeding, and can be severely toxic to the host (Klingauf, 1987a). The common response of plant tissue, when pierced by the stylet is to form necrotic spots, which can reduce product quality and value. Aphids also produce honeydew, a sugar rich excretion. Often leaves, fruit or vegetables become covered with honeydew which forms an ideal substrate for the growth of moulds (Hill, 1987; McKinlay *et al.*, 1992).

The major damage caused by *M. persicae* is however, due to transmission of viruses, both persistent and non-persistent forms. Aphids are very efficient virus vectors because of the needle like structure of the maxillary stylets that penetrate deep in to the plant tissue. Persistent viruses may come into contact with the insect just once, and from then on the aphid becomes a virus vector. The persistence is closely associated with the accessory salivary gland of the aphid vector (Garret *et al.*, 1996). *M. persicae* is an aphid species that can transmit persistent viruses. Non-persistent viruses can be transmitted only for a few hours after an aphid has fed on a diseased plant. In this case, the virus simply lies on the stylet and can be transmitted by all aphid species. Main viruses for the Solanaceae, (e.g. tomatoes, potato, pepper plants)

transmitted by *M. persicae,* are the persistent Potato leafroll and the non-persistent Potato virus *Y* and Tomato aspermy (McKinlay *et al.*, 1992).

1.1.3 Control of *M. persicae* and insecticide resistance

1.1.3.1 Chemical control

Aphids are generally controlled by insecticide applications. The main concern of growers is to avoid virus transmission. Soil applications, by systemic organophosphorus or carbamate insecticides, protect the early stages of the young plants. Foliar spray is also very important and again, organophosphorus or carbamate insecticides are mainly used. Other methods, adopted for suppressing the spread of viruses are, the use of mineral oils, which act in an unknown way, the use of pyrethroids, mainly with antifeedant activity, and last the use of combinations of mineral oils, pyrethroids and organophosphates (McKinlay *et al.*, 1992).

The development of resistance to these common insecticide groups by *M. persicae* appeared as a major threat to aphid control. Lately, the development of imidacloprid, a new insecticide of the chloronicotinyl group, very effective against homopteran species, provided both a solution to control *M. persicae* and a tool to develop resistance management for the particular pest (Elbert *et al.*, 1991; Elbert *et al.*, 1996; Nauen and Elbert, 1994; Nauen *et al.*, 1998a).

1.1.3.2 Insecticide resistance

The intensive use of insecticides was soon followed by the development of resistance in many insect species (Georghiou, 1994). In 1989, about 20 or so resistant aphids were reported and among them the most problematical were *M. persicae*, *Phorodon humuli* (Shrank) and *Aphis gossypii* (Glover) (Devonshire, 1989). *M. persicae* populations of moderate resistance first appeared in England in 1974, and in France in 1968 (Blackman *et al.*, 1978).

It is difficult to define resistance. Voss (1988), attributed the term resistance to the pest/product relationship, if the following criteria were met:

a) The product is recommended against the particular pest and has a previous successful performance

b) Failure to control the pest is not a consequence of incorrect storage, dilution or application, but due to environmental factors

c) The recommended dose fails to suppress the pest population below economic threshold level

d) Failure is due to an inherited change in the susceptibility of the pest to the particular product.

Development and function of resistance mechanisms

In glasshouses, *M. persicae* reproduce continuously by parthenogenesis and selection pressure from insecticides is very heavy throughout the year. The development of resistance mechanism is much faster compared with open field populations and the resulting resistance is also considerably stronger (Devonshire, 1989). The mechanism of insecticide resistance is based on the activity of a single enzyme, a carboxylesterase. There are two forms of esterases, E4 and FE4. The pure enzyme

shows an extremely low rate of hydrolysis of common insecticides. FE4 has a slightly faster hydrolysis rate compared with E4, but with little effect on the level of resistance (Devonshire *et al.*, 1983). The genes encoding the two esterase forms are very similar (approximately 5 kb) and the enzymes themselves differ only by nine amino-acid substitutions (Field *et al.*, 1993). The E4 and FE4 genes studied so far from various clones are identical (Field *et al.*, 1994). Resistance accrues from the amplification of the esterase genes and the large increase in the secreted enzyme that follows. The two esterase forms are rarely found amplified together in the same individual (Blackman *et al.*, 1999; Blackman *et al.*, 1996). These suggest a single amplification event for each gene followed by dispersal (Devonshire *et al.*, 1998).

These esterases confer resistance to a broad spectrum of insecticides (organophosphorus, carbamate and pyrethroid). Simple binding of the insecticide molecule to the catalytic centre of the enzyme provides a level of resistance because of the greater molar amount of esterase in the resistant aphids. The turnover of the reaction (hydrolysis) is slow, but with time low turnover can set free an amount of the enzyme, which can bind once again and remove even more toxicant. However, the initial amount of enzyme in the haemolymph is important for the survival of the insect (Devonshire, 1989). There are different levels of resistance, which are based on different levels of esterases presence in the insect's blood. Different levels of enzyme expression are regulated positively by gene amplification (multiple gene copies), rather than transcriptional control (Devonshire and Sawicki, 1979; Field *et al.*, 1988). The enzyme activity can be roughly assessed by the rate of hydrolysis of the 1-naphthyl-acetate to naphthol. Using this rapid and simple test, aphids can be characterized according to the level of enzymatic activity as: susceptible (S - none),

R1 (very low activity) R2 (medium) and R3 (high activity) (Devonshire *et al.*, 1986). In very resistant aphids the produced enzyme accounts for up to 1% of the total body protein. (Devonshire, 1989).

Two more mechanisms of target-site resistance have been identified lately for *M. persicae*.

A) The insecticide insensitive acetylcholinesterase is an important resistance mechanism, which had not been reported for *M. persicae* until 1990. Five years later this resistance mechanism was also reported in the UK (Foster *et al.*, 1998; Moores *et al.*, 1994). It confers strong resistance against organophosphorus and carbamate insecticides.

B) Knockdown resistance-type mechanism (kdr) is associated with modification on the sodium channels and confers resistance to pyrethroid insecticides (Martinez Torres *et al.*, 1998). Kdr provides stronger tolerance to insecticides compared to esterase based resistance.

Both cases of target-site resistance were found recently and they are strongly associated with the esterase mechanisms, suggesting that they evolved later and were also co-selected (Devonshire *et al.*, 1998; Field *et al.*, 1997).

1.1.3.3 Biological control

The development of insecticide resistance along with the concern about the environmental impact of current pest control practice, increased the attention on alternative forms of insect pest control (Charnley, 1997). Carver (1988) recorded an increased occurrence of introductions of aphid species in regions around the world in

the past few years. He suggested that there is an increased need for their control with natural enemies from their original habitat.

Van Driesche and Bellows (1996), described biological control, as the use of parasitoids predator, pathogen or antagonist populations to suppress a pest population making it less abundant and thus less damaging that it otherwise would be. Aphids do have a lot of natural enemies that can be used in biological control systems. Common aphid predators are various beetles (ladybirds, Coccinellidae), hoverflies (Syrphidae), chrysopids, anthocorid bugs and birds. Aphid parasitoids are wasps, belonging to the families of Aphidiidae and Aphelinidae (McKinlay *et al.*, 1992). Aphids also become infected by fungal pathogens. The most commonly encountered pathogenic species belong to the orders of Entomophthorales (Zygomycetes) and Deuteromycetes. Bacterial and protozoan infections have not been reported, but several baculoviruses and picornaviruses infect aphids (Latgé and Papierok, 1988).

Although various natural enemies have been tested as candidate biological control agents of aphids, very few have been widely accepted so far. For this situation Ramakers (1988) suggested the following considerations:

- despite insecticide resistance, chemical control of aphids is still effective by particular chemical insecticidal groups
- problems have been reported in attempts to incorporate aphid natural enemies in integrated pest management programs that involved use of chemical aphid control
- and last, aphid reproductive capacity is very high and only few natural enemies, except for micro-organisms have similar reproductive potentials. Successful biological control must therefore be based on repeated releases.

Aphid fungal pathogens have great potential for development as microbial control agents. They do not need to be ingested, as invasion normally occurs via the external cuticle, which makes them prime candidates for plant sucking insects, like aphids (Lacey and Goettel, 1995). The dramatic effects of natural epizootics on insect populations are also a reminder of the potential of fungal pathogens as control agents (Hajek and St Leger, 1994). Entomophthorales have been studied as control agents against aphids, but such studies are at an stage early compared to *Verticillium lecanii* (Ascomycotina) which has been developed as a commercial product (Charnley, 1997, Milner, 1997; Ramakers, 1988).

1.2 The pathogen *Verticillium lecanii*

1.2.1 The genus *Verticillium* Nees

The genus *Verticillium* contains a heterogeneous group of asexual fungi that includes important pathogens of plant, insects and nematodes. The genus was sub-divided into five sections (*Verticillium, Nigrescentia, Prostrata, Albo-erecta* and one residual section) mainly on the basis of morphological characteristics (Domsch *et al.*, 1980; Gams and Van Zaayen, 1982). The taxonomy of this group is still under review (Rowe, 1995). Bidochka *et al.* (1999) examined the nuclear rDNA phylogenetic relations of 18 isolates (from 13 species) in the *Verticillium* genus, from diverse econutritional groups (pathogens of insect, plants, mushrooms, nematodes and spiders). The phylogenetic data suggest that plant pathogens form a clade. Pathogens of insects like *V. lecanii* and *V. indicum* were present in divergent groups indicating that the ability to infect insects evolved independently many times. The same stands for the nematophagous *Verticillium* group.

There are nine entomogenous species of *Verticillium* listed by Gams (1971). Two of them (*V. lecanii* and *V. fusisporum*) have been isolated from *M. persicae* (Steenberg and Humber, 1999).

1.2.2 *V. lecanii* species complex

Verticillium lecanii was first described as *Cephalosporium lecanii* by Zimmermann (1898) and was later transferred to the genus Verticillium (Viégas, 1939). Taxonomically it is one of the most difficult species, with eight synonymous species named in 17 years (Evans and Samson, 1986). This is indicative of the variation within this species complex (Gams, 1971). Such intraspecific variation is highlighted by identification of isolate groups based on isoenzyme profiles (Jun *et al.*, 1991). Considerable genetic variation within the *V. lecanii* species complex has been shown by Kouvelis *et al.* (1999).

Description and characteristics

Verticillium is the genus of *V. lecanii*. Colonies of *V. lecanii* on malt extract agar (MEA) after 10 days at 20 °C can grow to 18 –22 cm diameter. They are white or pale yellow with a cottony-velvety appearance. Phialides are awl-shaped, very variable in size, on single or small groups of verticillate whorls on aerial mycelium. Conidia[1] are cylindrical to ellipsoidal with rounded tips. They are located in terminal head of slime on phialides (Figure 1.3). Conidia range in size from 2.3-10×1.0-2.6μm. Chlamydospores are absent (Domsch *et al.*, 1980; Gams, 1971). The fungus is also

[1] The term 'spores' will refer to conidia in this work

known as 'white-halo' because of the appearance of the white mycelium on infected insects (Figure 1.4-*A*) (Tanada and Kaya, 1993, p. 362).

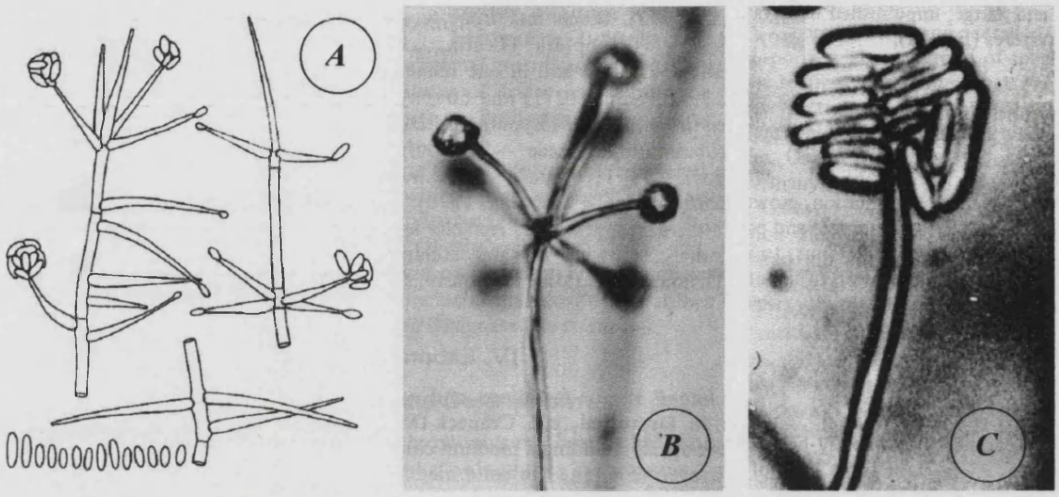


Figure 1.3 Conidophores, slime heads and conidia of *V. lecanii*.

A) Conidiophores, slime heads and conidia by Samson (1981)

B) Slimes heads *V. lecanii* (700×) (photograph by Hall, 1977)

C) Conidia inside slime head of *V. lecanii* (3000×) (photograph by Hall, 1977)

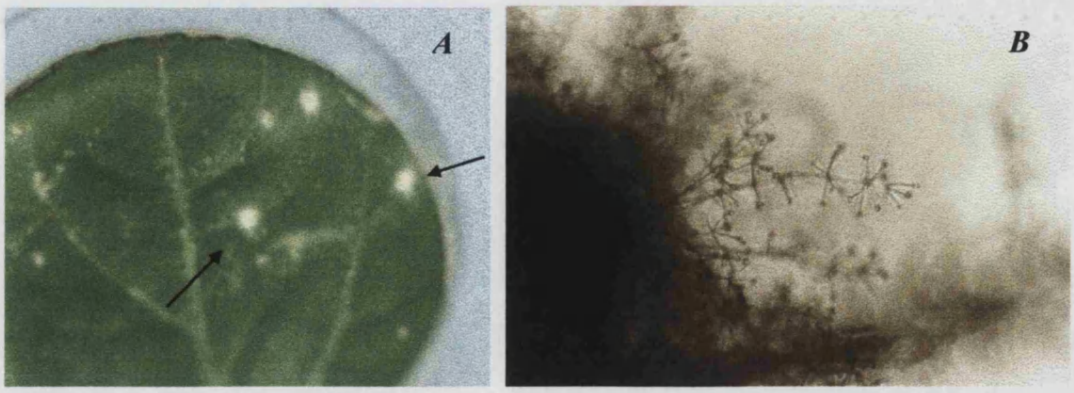


Figure 1.4 *V. lecanii* sporulating on *M. persicae*

A) Typical white coloured sporulation on *M. persicae* infected by *V. lecanii* as observed with naked eye (original photograph)

B) Sporulation of *V. lecanii* on *M. persicae* under light microscope (100×) (original photograph)

V. lecanii is one of the most important and common pathogens of scale insects and aphids in tropical and semitropical environments. It is less frequent in other orders of insects like Diptera, Hymenoptera, thrips and mites. Isolates have been reported to be pathogenic to the rust fungi, which cause plant diseases. Strains of *V. lecanii* are wide spread and occur under all climatic regions (Domsch *et al.*, 1980; Hall, 1981; Tanada and Kaya, 1993).

The fungus shows growth and spore germination between 15 and 30 °C with the optimal temperature at 23-24 °C. High humidity is required for spore germination. Temperature and humidity requirements are very important factors that should be taken into account for the good performance of the fungus in the field (Hall, 1977).

According to Hall (1981) *V. lecanii* generally showed good stability to continuous subculturing on artificial media. Also, no virulence increase was observed with passage through the host. The fungal conidia can be stored in the cold (2 °C) for 110 to 160 days. At temperatures around 20 °C the conidia can survive only under high humidity conditions. In dry conditions, more than 58% of the spores die in less than 24 hours. Spores attached on the parent mycelium can survive much longer.

V. lecanii has been used in crop protection in glasshouses with good results. Low-density aphid populations can be kept under control for the duration of the crop with just one spray (Hall and Burges, 1979). Under the name Vertalec™, it was first developed in the UK by Tate and Lyle. It is currently produced by Koppert B.V. (The Netherlands) and is used to a limited extent in glasshouses in the Netherlands

(Charnley, 1997). Vertalec™ is the only commercially developed myco-insecticide against aphids (Milner, 1997).

1.2.3 Infection process

Attachment of conidia

The first step in the infection process is the attachment of *V. lecanii* spores onto the aphid's cuticle. Ability to adhere to a particular cuticle mainly determines the host specificity. Sitch and Jackson (1997) demonstrated that *V. lecanii* showed reduced ability to remain attached on non-target invertebrates. This ability, depends on the spore structure and its physical and chemical properties. The processes involved in the attachment of pathogenic fungi to their insects host have been reviewed by Boucias and Pendland (1991).

V. lecanii spores disperse passively and attachment occurs mainly with water splashes or accidental contact with an insect host (e.g. sporulating cadaver, soil). In cases of artificial application direct contact between the host and the spore suspension is common, but limited, especially when the vegetation is dense. More important is the accidental pick up of spores from the spray residue on the leaves (Hall, 1981; Jenkins and Thomas, 1996).

Spore germination and penetration

After the attachment on the aphid cuticle the spore germinates. *V. lecanii* tends to grow considerably on the surface while attempting to invade the host (Drummond *et al.*, 1987). The signals involved to initiate cuticle penetration are not yet clear for *V. lecanii*. Appressorium formation prior to penetration is absent in *V. lecanii*, in contrast

to other common insect pathogens (*Metarhizium anisopliae* and *Beauveria bassiana*). Fungal pathogens can invade the host through body openings or through the cuticle, especially on soft bodied insects like aphids (Charnley, 1984). Cuticle penetration processes, by fungal pathogens, in general, require both mechanical pressure and enzymic degradation. A wide range of enzymes is involved in the cuticle degradation, mainly proteolytic, chitinolytic, and lypolytic/esteorlytic enzymes (Charnley and St. Leger, 1991). Penetration of the cuticle will allow hyphal growth in the procuticle and in the hemolymph.

Fungal growth and host defence

Once in the host blood, the pathogen can grow in the form of hyphal bodies and blastospores. Profuse fungal growth can cause death from starvation or physiological and biochemical disruptions (Charnley, 1997). *V. lecanii* produces insecticidal metabolites, mainly the cyclodepsipeptide toxin bassianolide, (also produced by *Beauveria bassiana*) which may also promote host death. (Roberts, 1981).

The main host defence reaction to the fungal invasion on a cellular level is encapsulation. The fungus is engulfed by granulocytes and the foreign body is lysed. Virulent isolates usually overcome encapsulation. The host humoral immunity defence mechanisms to fungi include the production of fungitoxic protease inhibitors and antifungal proteins (Hajek and St Leger, 1994).

Host death

Sporulation for *V. lecanii* occurs prior to the death of the host and can be observed on any body part, especially the antenna and the legs. The infected aphids have been observed to continue offspring production. In aphids infected with low doses, the internal organs were not invaded, even though fungal growth and sporulation was abundant on the whole body surface (Hall, 1981; Tanada and Kaya, 1993).

1.3 Pest control using invertebrate fungal pathogens: problems and solutions

Fungal entomopathogens are currently successfully used in commercial pest control. Nevertheless, there is a great effort, world wide, to improve further the quality and efficiency of these products according to the demands of the market. The main disadvantages of fungal pathogens in their use as pest control agents are their slow kill, the dependence on environmental factors (extreme temperature, humidity requirements, presence of pesticides, desiccation etc.) and the wide host range (hence they can have detrimental effects on beneficial insects) (Charnley, 1997; Lacey and Goettel, 1995; Milner, 1997). Some of the possible avenues for improvement of myco-insecticide efficacy in pest control systems are briefly described below.

1.3.1 Improving the isolates

Isolate selection

Isolation of new wild (or exotic) species or strains which are, more virulent, or more specific or with less stringent environmental requirements have been used for the development of better fungal insecticides (Van Driesche and Bellows, 1996). New

isolates can be selected based on laboratory bioassays on the desired hosts (Charnley, 1997). The term bioassay in the context of testing insecticides or bio-insecticides, as in this case, usually takes the form of assessing the mortality caused by the applied dose. Other responses, for example, in behaviour or fecundity can also be measured (Devonshire and Rice, 1988). It is also necessary to study the ecology of a new micro-organism before deciding on its suitability as a biocontrol agent. (D. Burges, unpublished).

Genetic improvement

Chemical mutagenesis, protoplast fusion and molecular techniques could be used to improve pathogen efficacy against target pests (Charnley, 1997; Tanada and Kaya, 1993). Protoplast fusion has been used to cross isolates of *V. lecanii* but only rarely do the recombinants produced have improved characteristics (Heale *et al.*, 1989). One example of successful construction of a mutated isolate was published by St Leger *et al.* (1996). Fungal pesticides have a low speed of kill (5-10 days), compared with most chemical insecticides (few hours). During that time the insect can cause serious damage to the crop. A 25% faster kill was achieved with a constructed isolate (*Metarhizium anisopliae)* which over-expressed a toxic protease (St Leger *et al.*, 1996).

Isolate Mixtures

Heale (1988) suggested the use of isolate mixtures with overlapping temperature optima to achieve pest control in a wide temperature range. *V. lecanii* isolates with different target pests have been used in mixtures. The aim being to control two pests

with a single application (Chandler *et al.*, 1993a). Unfortunately, the interaction between the isolates was competitive.

Culture conditions

Manipulating the culture conditions can improve the efficiency of a mycoinsecticide. The virulence of a fungus can be improved by manipulating the ratios of endogenous storage carbohydrates that affect spore germination. The properties of the growth media are directly related to the spore's storage carbohydrates (Hallsworth and Magan, 1994; Hallsworth and Magan, 1995).

1.3.2 Improving the efficacy of the application

Formulation and application

To date fungal insecticides have been improved by developing the technology of formulation and application (Prior, 1992). Better formulation enhances the stability of the product and gives a better longer shelf life. It can also give protection from UV radiation and prolonged viability after application and even gives the product new properties that could make it more effective (Bateman *et al.*, 1993; Moore *et al.*, 1992). Oil formulations are difficult with the lipophobic spores of *V. lecanii*, however successful attempts have been reported (Milner, 1997). Addition of various substrates (polysaccharides and phospholipids) in the formulation suspension was also shown to improve *V. lecanii* performance in field trials (Milner, 1997). At the moment ultra low volume (ULV) spraying is successfully used against locusts and grasshoppers (Sopp *et al.*, 1990). More recent developments, e.g., air assisted spraying[1], could possibly

[1] Fans are used to allow the spray of the abaxial surface of the leaves

provide a better crop coverage (Bateman, *pers. com.*). Other application methods of fungal pathogens include granule soil application, baits and traps (Charnley, 1997). In a novel application attempt, Butt *et al.* (1998) used honeybees as means to apply *Metarhizium anisopliae* against pollen beetles.

Combination with chemicals

Combination of microbial agents with other control tactics is one of the main tools in the development of an IPM approach. Cases of successful combinations of fungal insecticides with chemicals have been reported although the main issue is usually the incompatibility of the two control agents (see section 1.3.3). *Beauveria bassiana* has been used in combination with insecticides in China (Feng *et al.*, 1994). Synergy was detected in combined application of *Metarhizium flavoviride* with insecticides teflubenzuron (Joshi *et al.*, 1992) and cypermethrin (Sanyang and VanEmden, 1996). Increased efficacy of *B. bassiana* and *M. anisopliae* applications was also observed when incorporated with the chitin synthesis inhibitor dimilin (Hassan and Charnley, 1989; Jaronski and Goettel, 1997) and the insecticide imidacloprid (Quintela and McCoy, 1998b).

Combination with other organisms

Fungal insecticides and beneficial organisms can work together. Furlong and Pell (1996) have reported cases of parasitoids resistant to fungal infections. Rejection of infected hosts by parasitoids has been reported by Brobyn *et al.* (1988) and Fransen and van Lenteren (1993). Protection of the parasitised host (aphids and whitefly) from fungal infection has been reported only in late stages of the parasitisation (after 4

days) (Fransen and van Lenteren, 1994; Powell *et al.*, 1986). Combination of fungal pathogens with other microbial pathogens is not common (Charnley, 1997).

Manipulation of pest behaviour

Visual or chemical repellents and attractants, have been used to manipulate insect behaviour in order to protect crops (Foster and Harris, 1997). Some insecticides (imidacloprid) and oils (neem seed) can act as deterrents (Nauen, 1995; Nisbet *et al.*, 1994). A combination of a myco-insecticide with aphid alarm pheromone gave encouraging results (Griffiths and Pickett, 1987). Disturbance and enhanced movement in the presence of predators or parasitoids can increase the fungal mortality of pests (Furlong and Pell, 1996). Fungal infection itself affects the behavioural responses of the host (Hajek and St Leger, 1994; Tanada and Kaya, 1993). However, very few studies have looked on these effects in detail, particularly on *M. persicae* (Charnley, 1997).

1.3.3 Compatibility problems

The efficacy of fungal insecticides can be affected by other crop-protection practices and *vice versa*.

Chemicals

Common entomopathogenic fungi are not compatible with fungicides. The most common adverse effect recorded on fungal development was fungistasis, though in a few combinations the pathogen managed to overcome the inhibitory effects (Lagnaoui and Radcliffe, 1998; Majchrowicz and Poprawski, 1993). Commonly used herbicides had similar inhibitory effects on entomopathogenic fungi. An exception was the

herbicide lecanil, which showed less adverse effects. It is possibly compatible with most entomopathogenic fungi (Poprawski and Majchrowicz, 1995). More specific studies of the effects of pesticides on *Verticillium lecanii* were done by Hall (1981) and by Saito and Yabuta (1996). They showed that some pesticides had no effects on germination and myceliae growth of the fungus while others caused total inhibition. Nevertheless, Moorhouse *et al.* (1992) showed that fungicides and insecticides which are toxic to *Metarhizium anisopliae* during *in vitro* screening may in practice not be a problem.

Other organisms

Non target insects like beneficials (e.g. predators and parasitoids) or bees have been reported to be susceptible to entomopathogenic fungi (Milner, 1997; Quicke, 1997; Starý, 1988). Infected hosts can also sometimes transmit fungal disease to their parasitoids (Furlong and Pell, 1996). However, the safety of fungal insecticides is generally high, and natural barriers protect the non-target from the disease.

1.4 Aim

The methods listed above approach the same aim from different perspectives, which is to improve biological control and make it a more reliable, commercially acceptable and successful method of pest control. This is also the aim of this project. More specifically I have tried to contribute to improved biological control of aphids by studying the interaction between the pest *M. persicae* and the biological control agent, the fungus *Verticillium lecanii*.

The first target of the project was to study the effects of insecticide resistance on the susceptibility of *M. persicae* to the fungal pathogen (see Chapter 3). Clones with levels of resistance ranging from very low (susceptible) to highly resistant were tested. The behaviour of these *M. persicae* clones was also studied to see what additional effects high esterase levels have on the pest (see Chapter 4). These behavioural studies included the effects of *V. lecanii* infection on the host behaviour.

The studies on aphid behaviour were used to develop a behavioural manipulation method to improve the efficacy of the *V. lecanii* applications (Chapter 5).

CHAPTER TWO

Materials and Methods

This chapter describes general material and methods and basic experimental protocols. More specific techniques are described in the experimental chapters.

2.1 Materials

2.1.1 Pepper plants.

The green sweet pepper *Capsicum annuum*, v. California Wonder (provided by Moles Seeds) was used to maintain aphid colonies and carry out all experiments in this study.

About 20 seeds of pepper plants were sown in the nursery on a weekly basis. The compost type used was F2 (medium nutrient for seed sowing, Levington Professional). Young plants with about 2-3 cm stem length were pricked out, transplanted to individual pots using compost Q4 (multi-purpose compost, Vitax) and transferred to the main greenhouse (Figure 2.1).

The pepper plants were kept in a glass greenhouse at temperatures over 20°C. In case of lower temperatures, an air-heater was automatically switched on. During winter, when necessary, a few hours of additional artificial light was provided every day, to ensure quick growth (Camplex, Plantcare with 400 W Solarcolour HPS bolb, placed 1.6 meter from the greenhouse floor).

The plants were treated with nicotine fumes (Nicotine 40% - shreds, Dow Elanco) when needed to keep them free from wild aphid populations invading the greenhouse. Two infestations of mites were controlled by the acaricide Torque (0.5 g/l).

Figure 2.1. Pepper plants, *Capsicum annuum*, in the glasshouse in individual pots.

2.1.2 Aphid colonies

Four clones of the green peach aphid, *Myzus persicae* Sulz. (Homoptera: Aphidae), with different levels of insecticide resistance were maintained and used in the project. The clones, susceptible - US1L (S), medium resistance - 405D (R1), moderate resistance - T1V (R2) and high resistance - 794JZ (R3) were provided from IACR Rothamsted. Concerning the other two forms of target site insecticide resistance, none of the clones has the insensitive AChE mechanism, but the R2 and R3 clones have the kdr mechanism (Devonshire *pers. com.*).

2.1.2.1 The breeding cages

Large numbers of aphids were reared on potted plants mainly for experimental use. Wooden cages (1 m^3) housed 2 or 3 potted pepper plants. The cage sides were covered with thin cotton muslin, for ventilation and the tops were 4 mm clear plastic film to allow adequate lighting supplied from 3 fluorescent tubes (Philips TLD, 58W/29) 10 cm above the cage providing a light intensity of 2000 Lux. The cage doors were aphid-proof and secured with screws and most of them had acetated observation panels (Figure 2.2).

Eight cages were kept in an air-conditioned room at 21 ± 1°C and a photoperiod was 16 h light: 8 h dark. Water was supplied constantly from plastic trays placed under the pot. Good ventilation was maintained from the air-conditioner.

Each plant was thoroughly checked for any parasites or wild living aphids before being caged. One or two plants (per cage) were then infested with 10 adults from an

aphid clone. It took about 15 days for a new aphid colony to reach the numbers needed for a bioassay.

2.1.2.2 The Blackman boxes -maintenance of aphid clones stock cultures

Stock cultures of each aphid clone were maintained on small excised leaves according to the method of Blackman (1988). The advantages of this method are: low maintenance cost, small space allocation and highest reassurance of clone purity with re-culturing.

Blackman boxes were made from transparent plastic (7.5×4.5×2 cm) (Figure 2.3-*A*). A ventilation hole was made in the lid (diameter 1.5 mm) with a flamed cork-borer and was covered by very fine nylon gauze. A slide partition cut to fit the dimension of the box was placed 12 mm from the bottom of the box. The slide had a 3-mm-diameter central hole for the petiole of the pepper leaf to fit through. In this way an end-compartment was created and filled with a piece of polyurethane foam sponge.

The petiole of a small pepper leaf was passed through the hole in the partition into a vertical slit in the sponge. The sponge rested in water (0.5-1.0 cm of depth). The moist sponge preserved the leaf in good condition for about 15 days. A maximum number of 8 Blackman boxes were kept upright in a large polyester dish (Figure 2.3-*B*). The temperature was constant, at 21 ± 1°C and the photoperiod was 16 h L: 8 h D. Care was taken that the boxes had adequate light and ventilation.

Four of these boxes were prepared for each aphid clone by transferring 1 or 2 adult insects from another Blackman box of the corresponding clone. They were preserved

for no more than 15 days and in that interval a colony was established from the progeny of the aphids. These insects were then used for setting up new Blackman boxes or for infesting plants in breeding cages.

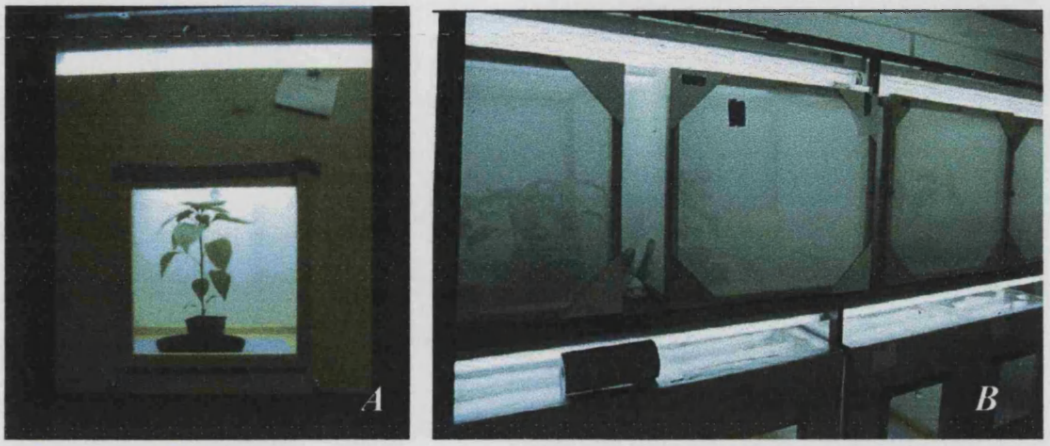


Figure 2.2. Potted plants in cages with glassed door (*A*) or door with thin mesh (*B*).

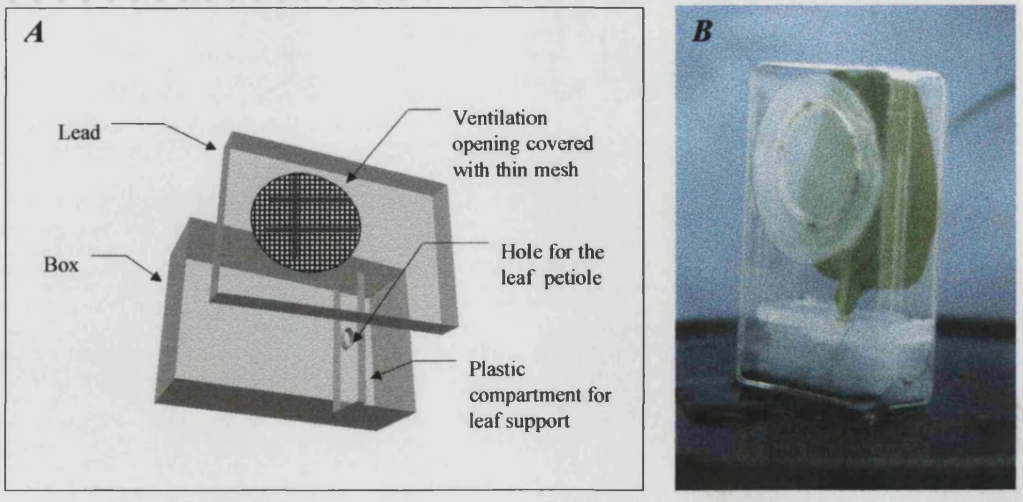


Figure 2.3. The Blackman box.

2.1.3 Fungal Cultures

The entomopathogenic ascomycete *Verticillium lecanii* (Zimmermann) was used in this study. The isolates used were: Vertalec (KV71) commercially used isolate from aphids; Mycotal (KV1) commercially used isolate from white-fly; KV22, KV42 and KV54 were provided by Koppert B.V.

Bactopeptone Agar (BPA) was used for routine fungal culture. BPA consists of 2% Malt extract (Oxoid), 5% Bacteria peptone (Difco Laboratories) and 2% Agar (Agar No3, Oxoid) (w/v). BPA plates were inoculated with 1 ml of spore suspension (10^6sp/ml) that was evenly spread over the plate. Cultures were incubated at 24°C and in the dark. Sporulation occurred after ca 8 days. These cultures were stored at 4°C and used as required over the next 2-3 weeks. New stock plates were established from the spore suspensions in 10% glycerol kept at -20°C.

2.2 Methods

2.2.1 The rapid test for esterase activity

Preserving the integrity of the four clones was crucial for the success of the project. Using the Blackman boxes for rearing the stock aphid cultures offered the highest security for the clones' purity. In addition to that, the esterase levels of the aphid clones were checked frequently using the following test. A rapid microplate assay was used for estimating total esterase activity in aphid haemolymph. The method employed was essentially that described by Devonshire *et al.* (1986).

Individual aphids were placed in 100µl of buffer (1.79 g $Na_2HPO_4.12H_2O$ and 0.68 g KH_2PO_4 in 500 ml - 0.02 M, pH 7.0 phosphate buffer) in the wells of a 12×8 microtitre plate. They were crashed with a multihomogeniser (Burkard Scientific) and 25 µl aliquots were transferred to a new microplate, using a multi-channel pipette, ensuring that the tips were washed between each transfer. The first column of the plate had just 25 µl buffer per well and acted as a blank.

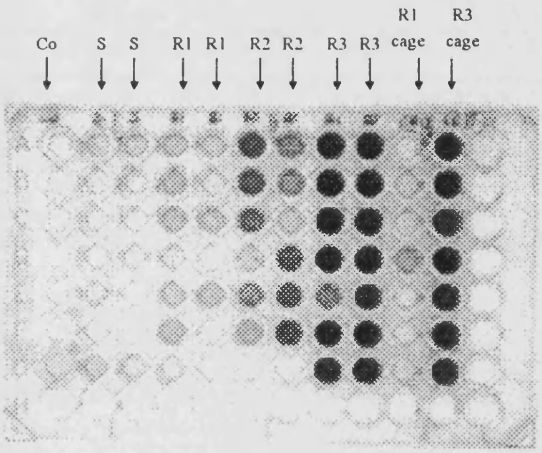


Figure 2.4 Typical microplate results from the rapid test for esterase activity.

The labels on top show the aphid clone tested in each column (Co for Control). Row H is empty. In some wells homogenisation was incomplete. This gave anomalous results, which were ignored.

0.5 ml of 30 mM 1-naphthyl acetate stock solution (558 mg 1-naphthyl acetate in 100 ml acetone) was made up to 50 ml with buffer to give a 0.3 mM solution. 150 µl were added to each well of the assay plate and left to incubate for 10-15 min at room temperature. DBLS solution was made up by adding a solution of 150 mg Fast blue B salt in 15 ml of water to 35 ml of 5% (w/v) of sodium lauryl sulphate. At the end of the 15-min incubation, 25 µl of the DBLS was added to each well, and the plate was left in the dark for 5 min. The plate was then be assessed by eye (Figure 2.4) or by using a microplate reader at 620 nm.

2.2.2 Spore suspension

The fungus *V. lecanii* produces hydrophilic conidiospores that are dispersed easily in water. The spores were harvested from the surface of an 8 to 10-day-old *V. lecanii* colony growing on a 9 cm BPA Petri dish. 10 ml of sterile single distilled water (dH_2O) was poured on to the dish using a 10 ml sterile plastic pipette (Sterilin). The spores were dislodged from the surface of the colony with a sterile hook. 1 ml of that suspension was diluted in 9 ml of dH_2O. The new suspension was diluted further to the desired spore concentration level, which was determined under the microscope using a haematocytometer at 400× magnification.

2.2.3 Repli Dish Bioassay

The bioassay described here was designed to estimate mortality of *V. lecanii* infected aphids after a 7 day incubation period. This method overcomes the problem of fast reproduction of the aphids that can make discrimination between the sprayed insects and the offspring difficult. The method has been described by Drummond *et al.* (1987).

Bioassay Plate preparation

Twenty-five compartment sterile square repli dishes were used. In each compartment 1.7 ml of molten sterile water agar (1% w/v agar in dH_2O) was poured. As soon as the water agar cooled down a square piece of pepper plant leaf, specially cut to fit the area of each compartment was placed on the agar with the abaxial surface uppermost (Figure 2.5-*A*). The water agar provided the leaves with the adequate water to keep them healthy during the experiment. Fluon® was applied on the edge of each

compartment, to prevent the aphids from climbing on the compartment's walls and escaping. The plate was usually left overnight for the Fluon® to dry before use.

The aphids were transferred to individual chambers (compartments) using a thin paintbrush (size N° 2). The brushes were sterilised before use by dipping them in 70% alcohol and left to dry for 1-2 min. To avoid injuring the insects and especially the mouthparts, the operator breathed lightly over the aphids. CO_2 in exhaled air caused the insects to withdraw their stylets from the plant. The aphids were then lifted from the rear. The plates were sealed with Parafilm M® (Figure 2.5-*B*) and placed in an incubator upside-down for 7 days (temperature 24±1°C, 16h L: 8h D). Adequate ventilation for each compartment came from two holes on the surface of the Parafilm® made with a needle. Parafilm® prevented the aphids from escaping and kept the relative humidity (%) in the compartment at dew point ~100% RH.

Assessment of mortality

The repli-dishes were left to incubate for 3 days (16h L: 8 h D, 24°C, ~100% R.H.) before observations started. This allowed minimal disturbance of the infected insects and in particular it ensured 100% R.H. during a long enough period for infection to occur. Plates were opened once a day and dead, missing and live insects were recorded. An aphid was considered dead from *V. lecanii* when the characteristic white-coloured sporulation on the insect's body was observed in combination with an inability to walk and absence of response when the insect was prodded with a fine seeker (see Figure 2.6). In case of doubt of the cause of death, the aphid was carefully examined under a dissection microscope.

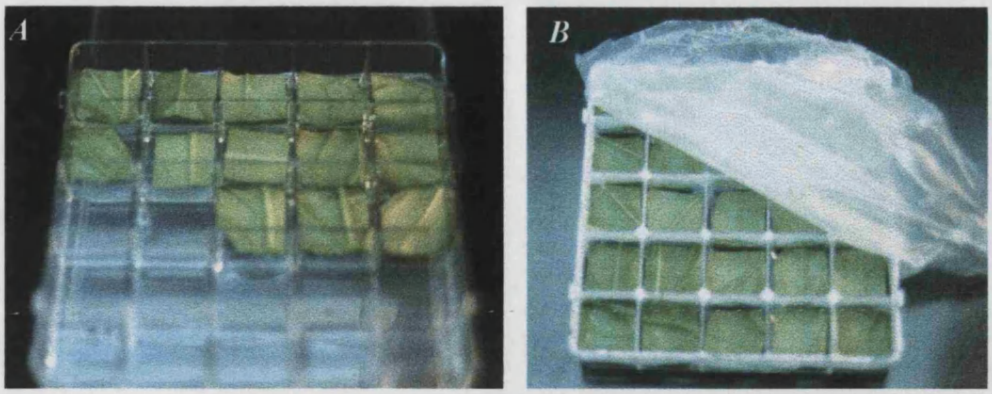


Figure 2.5 Repli dish under preparation (*A*) and finished and half-covered with parafilm (*B*).

Square pepper leaves, cut to fit the compartments were placed with the abaxial surface facing uppermost on a water agar bed

Figure 2.6 Adult *M. persicae* infected with *V. lecanii* - early sporulation state.

The characteristic white mould is visible with a naked eye. The infected aphid is darker and pink in colour. The hyphae will cover the aphid body completely in a few hours.

2.2.4 Spraying methods

In all three bioassay methods a Potter tower (Burkard Ltd.) was used to apply the fungal inoculum. The Potter tower is a means of applying a uniform and known spray deposit over a given area (Potter, 1952). Compressed Nitrogen gas was used as a propellent, at a pressure of 10 lb/in^2. Before and after use the Potter Tower was cleaned with 70% alcohol and then rinsed with sterile dH_2O. Similar application methods have been described by Milner and Lutton (1986) and Drummond *et al.* (1987).

2.2.4.1 *In situ* spore application

With this method the aphids were placed in individual compartments of the repli dish and sprayed *in situ*. Spores could attach to the insect cuticle either by direct impact with the sprayed spore suspension or by spore pick up from the leaf surface (secondary spore pick up). Spores and aphids were incubated together throughout the duration of the experiment.

1.5 ml of spore suspension at the desired concentration or sterile dH_2O for control treatment was transferred to an eppendorf tube with an automatic pipette. One repli dish was sprayed with the contents of one tube. The repli dish was covered with Parafilm® immediately after it was sprayed.

2.2.4.2 Direct Impact

The importance of the spore pick up by direct impact with sprayed spore suspension was evaluated using the following method. 30 aphids were placed in a 9 cm Petri-dish (12 ml 1% w/v water agar, 9 cm leaf disk with the abaxial surface uppermost) and left to settle for 12 -24h. The aphids were then sprayed with 1.5 ml of a spore suspension of known concentration ranging from 10^5 to 10^8sp/ml. The sprayed Petri dish with the aphids was left under a laminar flow cabinet for about 15 minutes to allow the spray droplets to evaporate to minimise contamination during subsequent transfer of the aphids with a paint brush to uninfected individual chambers. The aphids were incubated for 7 days (23±1°C, 16h L: 8h D) before assessing the mortality.

2.2.4.3 Secondary pickup

When an insect is walking on a previously infected surface there is a chance that spores will attach to the insect's cuticle. This is called secondary spore pickup. The importance of secondary pickup as a route of infection in aphids was studied in these experiments.

A leaf was cut to fit a 9cm Petri dish half filled with 1% (w/v) sterile water agar and with the abaxial surface uppermost. The water agar kept the leaf in good condition through out the experiment. The dish was sprayed with 1.5 ml of distilled water (control) or 1.5 ml of a spore suspension of desired concentration. The spore concentrations ranged from 10^8 to 10^5 sp./ml. The sprayed dish was left to dry for 15 minutes before any aphids were transferred on the leaf surface. This prevented aphids acquiring large spore doses by accidental contact with spore droplets during transfer

and also reduced the chances of contaminating the paintbrush used for moving the aphids around.

30 adult aphids were placed on the sprayed surface of the Petri dish with a fine sterile paintbrush and were incubated for 24 h (unless otherwise stated in the results) (conditions: 23±1°C, 18h L: 6h D). After that time interval the insects were transferred, with a sterile brush, to individual uninfected square chambers of a repli-dish. The paintbrush was surface-sterilised in 70% alcohol. The repli dishes were prepared as described in the 'Repli dish bioassay' section. The aphids were incubated for 7 days at 23±1°C (16h L: 8h D) and the mortality was estimated on the last day by counting the infected insects.

Modification for secondary pick up experiments with imidacloprid infused leaves

The Petri dishes used were of a 5 cm diameter instead of 9 cm. The dish was changed for two reasons: a) with a smaller dish it was easier to obtain a leaf disk completely covering the water agar bed, thus eliminating certain kinds of variation and b) for consistency with the method followed in the experiments studying the behaviour on imidacloprid treated leaves. The preparation, spray deposit of fungal spores on the leaf surface and drying of the dishes were the same as in the standard experiment.

2.2.5 Behaviour experiments

The behaviour of the aphids was studied using a time-lapse video recorder that compressed the time and the duration of the observations. Two basic methods were adopted for experimentation: a) Observing the aphids and recording the events using a

computer program, b) Using a computer program that was able to track the insects and find their paths. Both methods are described below.

2.2.5.1 Set-up for observation experiments

The experiment was split in two sections. First the aphids were recorded on tape, the tape was then played back and the behavioural data were collected.

The basic elements of the set-up for recording the aphid behaviour are displayed in Figure 2.7. The camera, a monochrome Panasonic WV – 1850/B with a close up lens, was positioned so that it could record the aphids on the underside of the leaf. The camera was connected to a time lapse video cassette recorder (VCR, Sanyo TLS-1500P). The VCR compressed 6 days of continuous recording onto a 3-hour tape. The TV screen was a monochrome Sony PVM-201 CE, and it was used for adjusting and focusing the image of the observation plate while filming. The TV was also used for observing the aphid behaviour when the tape was played back. The lighting came from two cool light sources that prevented the temperature from rising near the observation area. A thermometer attached to the stand monitored the temperature near the observation area. The stand was a transparent plastic box (16×11×2cm) turned upside down and firmly attached to the camera's lens. The stand had the proper objective distance for the close up lens (2cm) and at the same time protected the lens surface from the honeydew produced by the aphids during the recording period.

The observation arena was a 5cm Petri dish, half filled with 6 ml of sterile, distilled water agar (1% w/v) with a pepper leaf disk applied to it. The leaf disk was placed

with the abaxial surface facing uppermost. 5 aphids were placed on the surface of the leaf and then the Petri dish was placed upside down on the stand.

The recording started as soon as the lights were adjusted, and the image of the observation disk was centred and focused. The photoperiod was 16h L: 8h D and the room temperature constant at 23 ±1 °C, controlled by an air-conditioner.

After the recording was finished the tape was played back and while observing the tape the computer program 'Observer', was used to record the events. The aphid behavior was categorised as, feeding on the leaf, moving about, producing drops of honeydew and giving birth to offspring. The data were analyzed using the Microsoft Excel program.

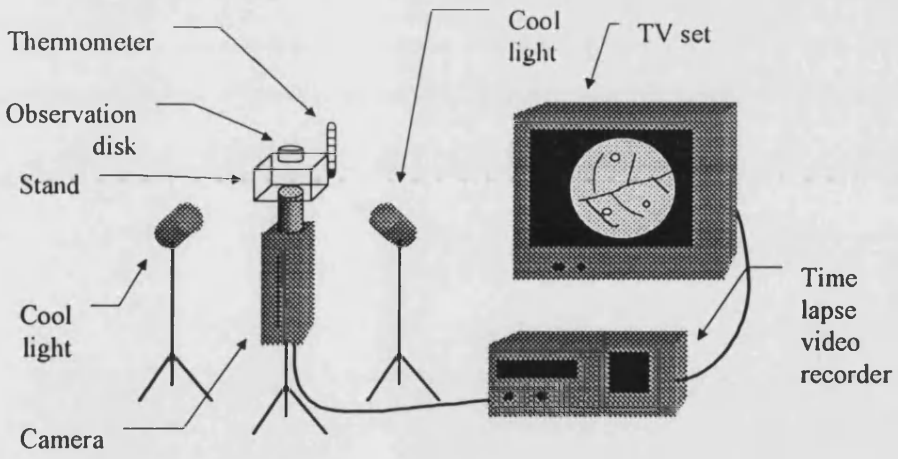


Figure 2.7 The filming set-up for observing aphid behaviour.

2.2.5.2 Tracking set up

The method of filming aphid behaviour was a slight modification of that described in 2.2.5.1. A computer was used to analyse the images. The computer program was very sensitive to alterations to the image lighting and vibration for the outside environment. Care was taken that none of the above was occurring during filming.

Filming

The tracking set up is shown in Figure 2.8. The equipment was placed in an incubator with constant temperature of 24 °C and photoperiod at 16h light: 8h dark. The camera used was a Panasonic NV-SX30B. The stand was placed on cork to absorb any vibrations caused from the motor of the incubator. 6 fluorescence tubes (GE, F8W/35) were placed few cm above the arena providing illumination of 1200 Lux. The arena was a 5cm Petri dish (deep) with 6 ml of water agar (1% w/v) and a leaf disk placed on top. Tracking was done from videotapes. Unfortunately analysis of time lapse video tapes was almost impossible, except in the case of the lowest time lapse, mode 12 or 24 (15 or 27 h real time (respectively) = 3 h time lapse recording). The basic problem was that the walking speed of the insects in time lapse tapes was too high for the computer to keep track of the individual insects. The computer was equipped with an Intel Pentium 166MHz processor and 64Mb of 72 pin EDO RAM.

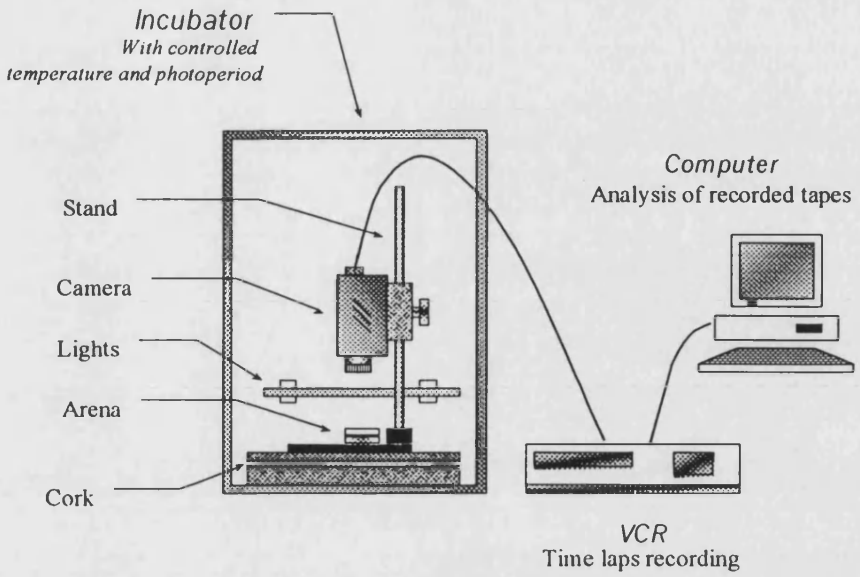


Figure 2.8 The filming set up and the equipment involved for tracking aphids.

The basis of track analysis program

The method was developed by I. Couzin (*thesis in prep.*). The analysis was done in 2 steps. First the program named 'AphidTrack' was used to find the *x-y* co-ordinates of the aphids on the screen with time. Another program 'AphidData' was used to plot the trajectories and extract biologically meaningful values from a series of numbers (*x,y* co-ordinates) produced by the 'AphidTrack' program.

First the computer was connected to the VCR through a Matrox Meteor frame grabbing computer card. The analogue image of the videotape was digitised to a 768x576-pixel greyscale image. Initially the development of the tracking program 'AphidTrack' that would be capable of analysing the images grabbed from the camera was impossible because of the complexity of the leaf surface used as the arena background where the aphids were recorded. The leaf disk had to be used as the arena surface because it was the set up closer to both natural field conditions and the bioassay experiments. Also it provided the food source that insects were cultured on.

The problem was solved by using a 'background subtracting' technique that would remove that unwanted complexity. The tracking program was finally developed based on the resulting image of this technique.

The developed 'background subtracting' technique was based on the fact that the images were made by 768x576 pixels and every pixel has a greyscale value from 0 (black) to 255 (white). An algorithm was build and it was possible to subtract one image from another. What the algorithm was actually doing was subtracting the pixel values of respective pixels on the image. The pixels in the resulting image (difference image) would be the absolute result of the subtraction (Figure 2.9). If the two images were the same the resulting image would be black (zero value). Any differences would appear as bright dots.

Subtracting pixels -Numeric example from Figure 2.9*:*

{pixel (*B, I*) captured image (value **200**)} - {pixel (*B, I*) background image (value **5**)} =
{pixel (*B, I*) resulting image (value **195**)},
In summary: 200 - 5 = **195**

In practice, the arena without aphids was recorded and that image was used as a reference background image for the analysis. From that point onwards the rest of the images were subtracted from the background image and the analysis was done on the resulting image. The complexity of the leaf surface was removed leaving only the aphids as white dots on a black background and a small amount of filming noise (Figure 2.10).

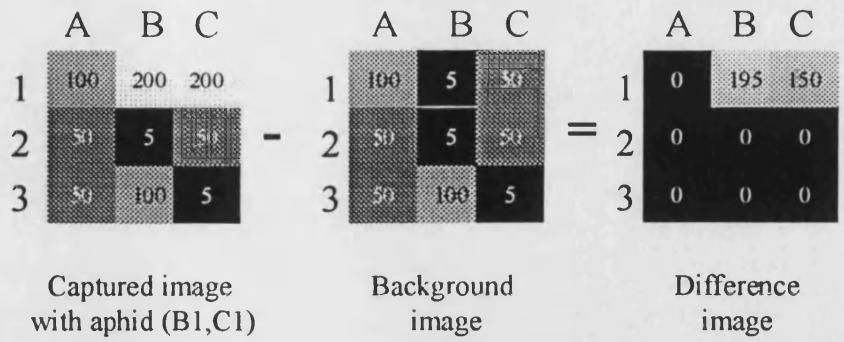


Figure 2.9 A simplified way of demonstrating Background subtracting.

Using the reference (Background) image (3×3-pixels) is possible to reduce the complexity of the captured image and produce a simplified 'Difference image', which can be analysed by the computer. Threshold values are displayed in boxed areas. The system is using absolute difference values.

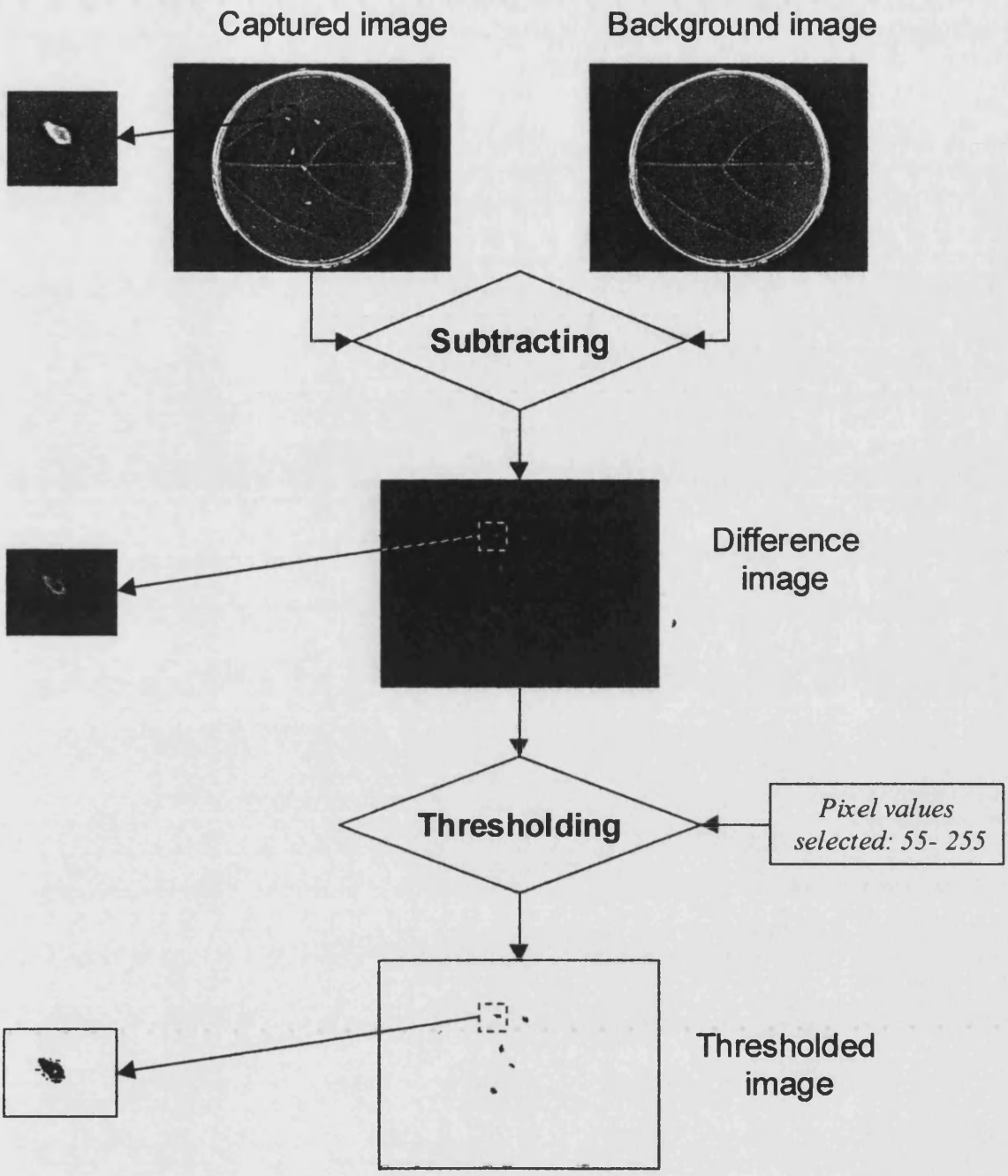


Figure 2.10 Steps followed by the 'AphidTrack' program for the image analysis

The background image is subtracted from the captured image, the difference image is thresholded, and the selected pixel values produce five blobs, representing the aphid positions on the image. The program will record the *x-y* co-ordinates, the size each blob, and the time that the image was captured.

The computer was programmed to find pixels with threshold values higher than a specified value usually 45 - 65 (Black=0, White=255). The selected pixels produced the thresholded image. From experience an adult aphid could not be smaller than 20 pixels so any areas smaller than that were rejected as filming noise. On screen the aphids look like aphid-shape black blobs. The centre of gravity (x, y co-ordinates) and the area of each blob (aphid) as well as the frame number were recorded on an array. A second array kept record of the time that each frame was captured. The tracking session could last from a few minutes to few hours, and about 6 to 10 frames were analysed per second depending on the settings. The data on the arrays were saved to the hard drive as text files (specially formatted, sample text in Appendix 2).

'AphidData' was able to read the text-files and with a simple biological rule was able to tell apart the aphids. The rule had two parts. The first was that the aphids could not cover a distance longer that their body length per frame (20 pixels in 1/6 of a second)[1] (Tourtellot *et al.*, 1991). The second part referred to the points within that range. The point closer to the previous position was the new position of the same aphid. This way a new text file was produced with the x-y co-ordinates of a single aphid with the time (sample of the text file format in Appendix 2).

From the trajectories it was possible to find and plot the path that the aphids followed during the recording. Also biological values like distance covered, and average speed could be calculated.

---

[1] The computer could calculate length only in pixels. The real length could be found later with calibration.

Allowing for edge effect anomalies

The centre of gravity of the blob (aphid body tracked by the program) representing the position of the insect on the arena continually flipped between positions (1-2 pixels) from frame to frame during tracking although both the image on the video screen was stable and the aphid was still. This problem was caused by the so called the 'edge effect'. When filming, the limits between objects are not strictly defined, and the edge of the objects could have a few pixels difference between frames. That is hardly visible and has very little to do with equipment quality. However, it was detected by the tracking program, resulting eventually in a false 'distance covered' value.

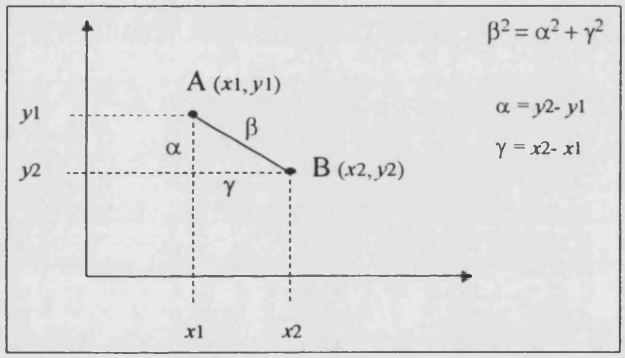


Figure 2.11 Geometry used to calculate the distance covered by an aphid.

The insect moved from position A to B. The distance covered is β and it can be calculated from the (x,y) co-ordinates of A and B as shown in the formula.

The data were processed by Microsoft Excel and the distance covered was calculated using very simple geometry as shown in Figure 2.11. An algorithm was build to detect real mobility of the aphids from the edge effect anomalies. The algorithm gave a '1' value for distance covered when movement was detected and '0' value when the edge effect was detected (see Appendix 2). The distance array was multiplied with the array produced by the algorithm and the resulting array contained only the distance-covered when the insect was walking.

Filming during the dark phase

Studies have shown that insects in general are sensitive to light in the blue-green region of the spectrum and insensitive to red. In some species, sensitivity to the red has been detected (families of: Hymenoptera, Lepidoptera, Odonata, Coleoptera) however it has never been recorded such a case for any Hemiptera species (Goldsmith and Bernard, 1974; Menzel, 1975; Saunders, 1982). Spectral sensitivity has been studied in detail on the aphid *Megoura viciae*. The insect showed maximum sensitivity in the blue (450 - 470nm), fifty times less sensitive at 365nm or in the green at 550nm, and completely insensitive in the red (>550nm) (see Appendix 3 for related table and figure from Saunders (1982); p. 186 - (quote Lees, 1966; Lees, 1971)).

The set up was modified so as to allow filming during the dark phase. A dark room red safe light (Kodak No 1) with a cut of point at 610nm (Figure 2.12) was placed in the filming area facing the arena. The filter allowed only 10% transmittance in the spectra region of 610nm and over. The lamp of the safe light was replaced with a 40W (Philips) to allow adequate light for the camera (3lux). The safe-light was turned on automatically as soon as the day-time lights were off to avoid overheating of the incubator. A similar set up for night filming (time lapse) of *M. persicae* was used by Nauen (1995). The camera was sensitive enough to record a very dark image with the aphids as lighter coloured blobs (Figure 2.13). Continued tracking of the insects was possible when the threshold value setting of the 'Aphidtracking' program was as low as 25 -28 (0 = white, 255 = black).

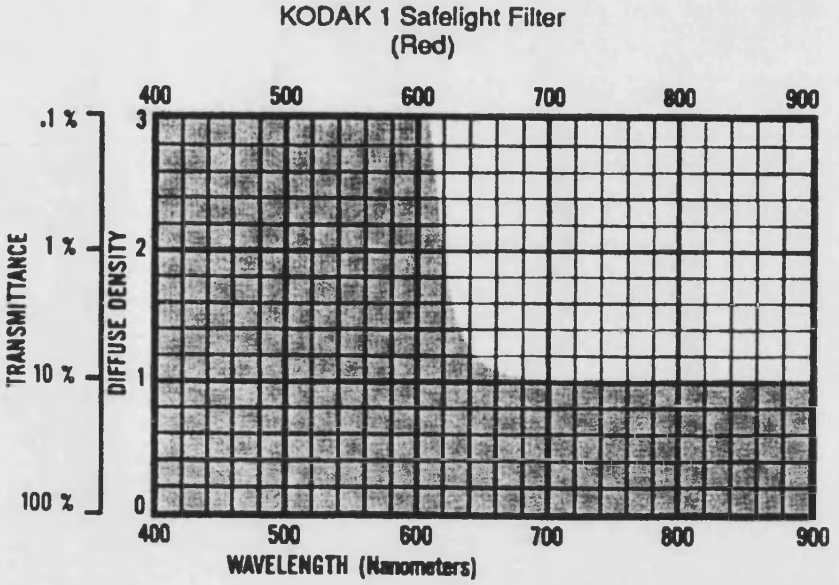


Figure 2.12 Transmittance of the Kodak 1 safe-light filter

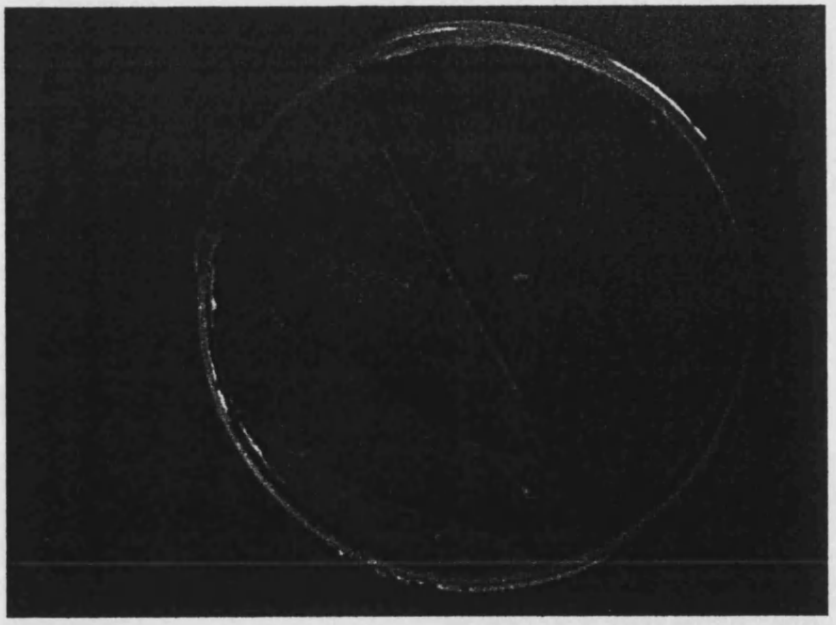


Figure 2.13 Image of an arena with aphids captured during the night filming period.

Dual Filming

In order to reduce variability between experiments control and treated insects were filmed at the same time. Simultaneous filming was achieved by using a second camera and time-lapse video recorder (Figure 2.14). The camera was the same Panasonic model and was placed parallel to the first one in the incubator. The filming areas did not overlap.

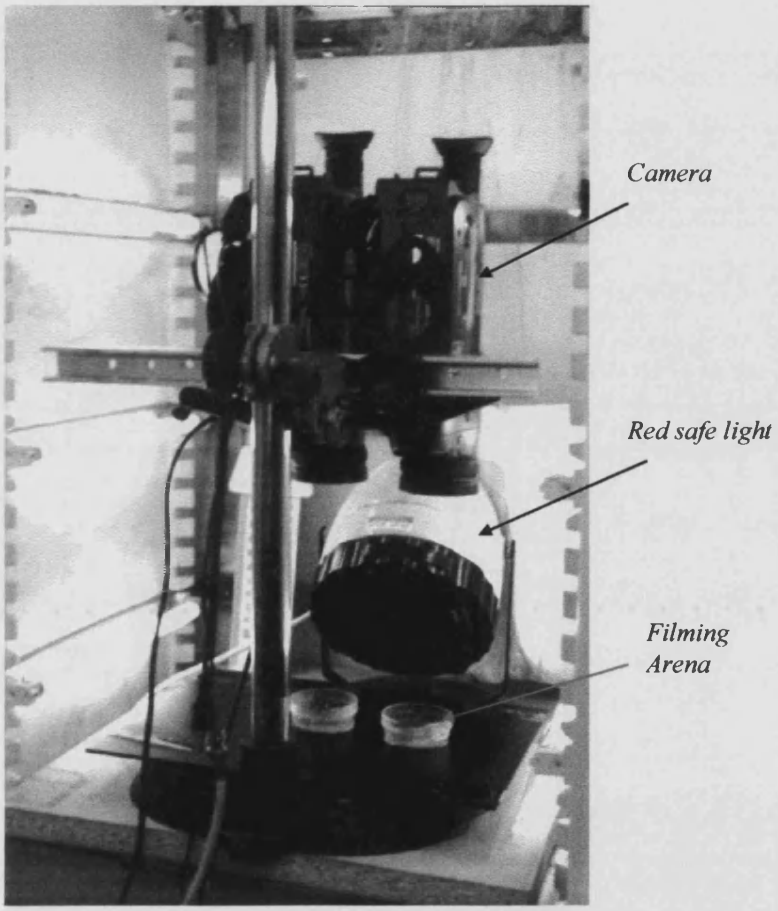


Figure 2.14 Filming set up with two cameras and red safe-light as it was set in the incubator during the experiments.

2.2.6 Data analysis

2.2.6.1 Statistics

The program Minitab® for Windows (12.22) provided the analysis environment for running statistical tests (*t*-test, regression analysis, ANOVA, Chi-square test, Mann-Whitney *U*-test, Kruskal-Wallis test etc.).

2.2.6.2 Correction for control mortality

In case of control mortality the estimated mortality (%) was corrected using Abbot's formula (1925):

$$M_c = \frac{M_p - M_t}{100 - M_t}$$

M_c = corrected mortality
M_p = estimated mortality
M_t = control mortality (all expressed in percentages)

2.2.6.3 Determination of the median lethal concentration

In dose response experiments the median lethal concentration[1] (LC_{50}) was estimated by Genstat procedure library, using probit analysis. The details of the probit analysis program can be found in Appendix 1.

The dose response curve of the logarithm of the spore concentrations against percentage of mortality is usually a normal sigmoid curve which can be converted to a

[1] The spore concentration required to kill 50% of the treated population.

straight line by transforming the percentages to probability units (probits). This method places greatest weight on the more reliable values in the region of 50% kill. The resulting linear equation allows determination of LC_{50} and probit slope with known accuracy (Devonshire and Rice, 1988).

LC_{50} was used to compare the relative efficiency of different fungal isolates or different aphid strains. The slope of the probit line gives a measure of the spread of the response.

Running the Probitan program it was possible to determine if the responses of experiment treatments were significantly different. The program used three linear models. For the first model the results from all treatments were all fitted in one common linear equation. For the second model the results from the treatments were fitted in parallel linear equations (same slope) and the third model used linear equations with independent slopes for each treatment. The program gave as an output the LC_{50}, estimated standard errors and degrees of freedom (*df*) and deviance for all models. If the deviance was significant then the results did not fit that model and it was rejected.

The conclusions for the data depended on the model that fitted the results best. If the first model was accepted the dose responses of treatments were considered similar. If either of the other two models was accepted then one of the treatments was significantly different from the other(s).

CHAPTER THREE

Comparative studies on susceptibility of *M. persicae* clones to *V. lecanii*

3.1 Introduction

Widespread use of insecticides has lead to the development of resistant clones of the peach aphid *M. persicae*. Resistance is associated with increased production of E4 or FE4 esterase, an enzyme that detoxifies the insecticidal esters, before they can affect the insect's nervous system (Devonshire, 1977; Devonshire and Moores, 1982). It is now established, that different levels of insecticide resistance results from the level of amplification of one ester gene (Field *et al.*, 1988). Extremely resistant aphids have up to 60 copies of the structural gene, and devote approximately 1% of their protein production to this enzyme (Devonshire and Field, 1995).

Devonshire and Sawicki (1979) suggested that this was a case of evolution by gene duplication without subsequent mutations. Indeed, restriction mapping and PCR sequencing of the esterase genes, from 20 resistant aphid clones, from different

geographical origin, showed absence of variations between the genes, and slight differences were observed with the unamplified esterase gene from susceptible clones (Devonshire and Field, 1995). Also Field and Devonshire (1997) presented evidence that the E4 and FE4 genes are part of the same gene family. Apparently, this mechanism of insecticide resistance is well conserved, and varies very little between clones. In addition, Al-Aboodi and ffrench-Constant (1995) confirmed absence of genetic variation between resistant aphid clones for *M. persicae*, supporting the initial assumption of absence of mutations.

Bowers (1992) suggests that the cost of chemical defence has both a physiological and an evolutionary perspective. From the physiological point of view, there is a 'direct' energetic cost to *de novo* synthesis of defence chemicals due to: *a*) resources utilised to produce the compound (other wise to be used for growth), *b*) enzymes needed to catalyse the reaction for the synthesis and, *c*) prevention of autotoxicity (storage or removal from the system). The 'indirect cost ' from the evolutionary perspective, associates the levels of chemical defence with fitness difference correlates. Growth rate, weight and fecundity have been used as supposed correlates of fitness. Insect species, (mainly from families Coleoptera and Lepidoptera), have been investigated for evidence of cost of the chemical defence (Bowers, 1992, Table 7.3).

Chemical defence has been exploited by insects for protection from parasitoids and predators (Bowers, 1992). Although this is not the case for resistant *M. persicae*, there is a form of chemical defence (resistance) against insecticides, with *de novo* synthesis of the esterase, acting as protector from intoxication. Crow (1957) suggested that in

absence of insecticide there is a selective cost for resistance. In the present work, a hypothesis was made that the cost of the large amounts of esterase produced by insecticide resistant aphids might be increasing the susceptibility to the fungal infection. It is well established that various types of stress predispose insects to disease (see Charnley, 1997).

Preliminary experiments

Occurrence of variability in biological assays and the reasons for it, have been discussed many times (see e.g. Clark, 1933; Finney, 1964). Variability still occurs even if the experimental conditions are controlled. However, it is important to reduce variability to a minimum, in order to reveal differences between treatments.

The preliminary experiments were developed exactly for that reason. They are directed to investigate very basic principles or relationships as aspects of the bioassay. The effort is focused on relationships between spores applied and spores deposited, using the Potter tower application system and between spores deposited and spores germinated on aphids.

The Potter spray tower (Burkard Ltd.), is used to apply spores directly to insects or leaves. The volume and dose administrated is known, but only a fraction will actually hit the target (insect), or the leaf (approx. 10%) (Fransen, *pers. comm.*).

A vital staining technique developed by Drummond and Heale (1985) enabled observation of conidiospore germination and growth, *in situ,* on aphids. Studies on *V. lecanii* spore germination by Sitch and Jackson (1997) using a similar technique,

under laboratory conditions, revealed a good spore germination (%) on *M. persicae*. Similar germination experiments were developed to establish a relation between the number of spores that hit the target, and the number that eventually germinate.

It is a common practice in *V. lecanii* bioassays, to apply the spores in Triton X-100 water suspension (Chandler *et al.*, 1993b; Hall, 1979). Additionally, Hall (1977) studied the effects of different Triton X-100 concentration on the aphid viability in dipping experiments. He found that *M. persicae* was tolerant up to 0.5% Triton X-100 concentrations. However, aphids treated with high Triton X-100 concentrations, showed signs of irritation and restlessness. Triton X-100 is not always used as an additive in the water spore suspensions (Schreiter *et al.*, 1994; Yokomi and Gottwald, 1988). For this reason, a preliminary experiment was designed, to investigate any effects of 0.02% Triton X-100 on the efficiency of *V. lecanii* conidia application in the bioassays. The concentration 0.02% Triton X-100 has been considered as standard for aphid bioassays (Hall, 1976a).

Milner and Lutton (1986) established in laboratory experiments, the importance of secondary pick up of conidiospores from *V. lecanii* infected leaf surfaces for the control of *M. persicae*. They also illustrated the requirement of prolonged exposure periods, and high humidity conditions, for both the successful transmission (secondary pick up) and germination of the conidiospores (experiments carried out on whole plants). Similar experiments were developed, to study the requirements of the 'secondary pick up' experiments, in the currently developed bioassay set up.

3.2 Materials and Methods

3.2.1 Materials

Uvitex BHT (CIBA GEIGY) is a fluorochrome, that attaches to cell walls of the conidia, and fluoresces under UV-light. Staining of the spores, allows their identification post inoculation on the aphid body under a UV microscope. When a spore germinates, the Uvitex BHT stain is also present on the germ tube. Uvitex BHT at the concentration used (0.1%), does not affect spores germination of *Metarhizium* spp. (Bath, 1997). Triton X-100 (iso-octylphenoxypolyethoxyethanol) was supplied by BDH. All experiments were carried out using *V. lecanii* isolate KV71 that forms the active ingredient of Vertalec.

3.2.2 Methods of preliminary experiments

3.2.2.1 Estimating the body surface of an aphid

The size of the aphid body varies, but it is possible to estimate the average body size, with the aid of image analysis.

Image analysis

The aphids were illuminated from underneath, using the light of an Olympus microscope. The resulting image, consists of a dark object (aphid), on a bright background, as shown in Figure 3.1. A video camera (Panasonic, WV-1850/b) was fitted to the microscope, using a special video-adapter ring. Using the same PC described in the 'Tracking aphids' section, in Chapter 2, the image from the camera was captured and digitised. The image was analysed by program Visilog ™ (Noesis),

and the area of the aphid was calculated in pixels by thresholding (selected pixels with value < 130). Finally, using a calibration slide, the pixels were transformed in mm^2 (235 pixels = $1mm^2$). In this way, the body surface was estimated for each aphid that was used in the 'Dose response' experiment in the following section.

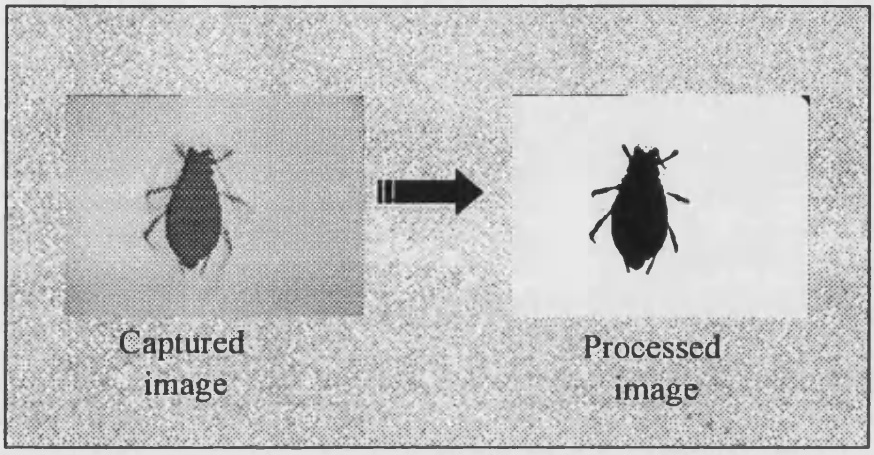


Figure 3.1 Image of and aphid body captured and analysed by computer.

The captured image is thresholded (selected values < 130) and the surface of selected pixels on the processed image (black pixels) form the surface of the aphid.

3D model of aphid body

Because the aphids are not 2-dimensional, as it was assumed in the previous experiment, a new method was developed for estimating the aphid body surface. The first step in building the 3D model was to find a geometrical shape, to fit the aphid body and to simplify the calculations. The double cone fitted well the lines of the aphid body (Figure 3.2). The final shape of the double cone is shown in Figure 3.3. The measurements needed to build the model were the aphid body length, the distance between the eyes and the maximum width of the abdomen. Ten adult aphids were killed with chloroform, and were immediately transferred under a calibrated light microscope with a scaled eye piece lens, to measure the specific dimensions.

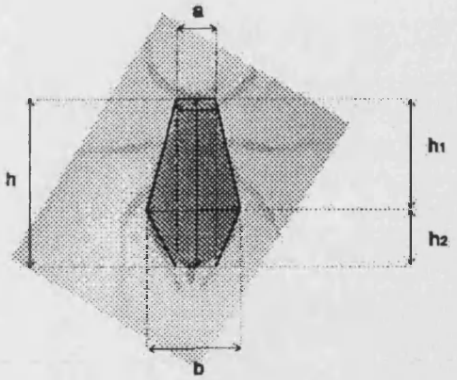


Figure 3.2 The conical 3D model fitted of the aphid body.

For simplification the shape of the aphid body is assumed to be the double cone. (*h*) is the body length, (*a*) is the distance between the eyes and (*b*) is the wider measure of the abdomen. (*h1*) and (*h2*) are not needed in the calculations (see Figure 3.4).

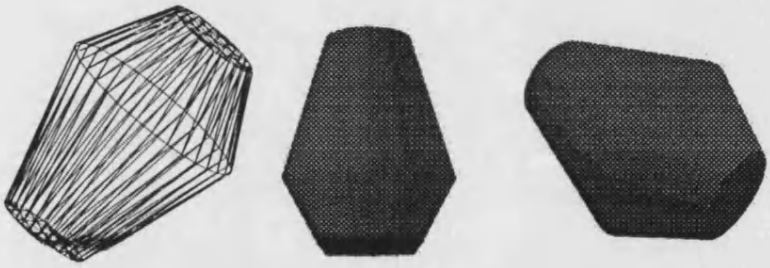
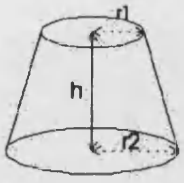


Figure 3.3 Final forms of the 3D 'aphid body' model.



Surface of the double cone:

$$S = s1 + s2 = \pi(r1+r2)h_1 + \pi(r1+r2)h_2$$
$$= \pi(r1+r2)(h_1+h_2)$$
$$= \pi(r1+r2)h$$

Figure 3.4 Geometry used to calibrate the cone surface.

Two simple cones make the double cone of the model. Adding their surfaces (s1+s2) will result to the surface of the double cone (S) as shown by the formula. The double cone has the same surface as a simple cone of the same height (h).

The surface of the double cone is equal to the sum of the two surfaces of the individual cones. The geometry and formulas used are shown in Figure 3.4. Looking carefully in Figure 3.2 and Figure 3.4, it is possible to relate formula parameters, to aphid measurements ($r1 = a/2$ and $r2 = b/2$). An estimate of the average body surface of an aphid will result from that formula.

3.2.2.2 Calibrating the Potter Tower

Preparation and observation of Uvitex BHT stained spores

Spores were harvested from a 9-day-old colony of *V. lecanii*, as described in the relevant section in Chapter 2. 1 ml of the spore suspension, and 1 ml 1% Uvitex BHT were added, in 8 ml of sterile water, making up to 10 ml of a new suspension, of 0.1% Uvitex BHT, and of approximate concentration of 10^8sp/ml. The suspension was then incubated for 1-2 hours. The spores were washed twice, by spinning down (centrifugation at 5000 rpm for 10 min), removing the supernatant, and re-suspending them in water. Removing the Uvitex BHT stain from the suspension was important, for avoiding background fluorescence, when observing under the Olympus BH2 microscope. A range of spore suspensions was prepared, and the spore concentration was estimated under the microscope (400× magnification), using a haemocytometer.

The Olympus BH2 microscope was equipped with an episcopic fluorescence illuminator, supplying UV light a from 100 W mercury lamp. Excitation wavelength was 350 nm, dichroic mirror at 400 (V) and barrier filter L-435.

Testing the homogeneity of the spray deposit

In these experiments, the relation between the distance from the centre of the sprayed area and the spore deposit was investigated. The most distant spray point was considered to be the corner of the bioassay repli dish; the largest item to be sprayed under the potter tower. On a lid of a repli dish, 5 numbered cover slips were placed as shown in Figure 3.5-*A*, on the corners, and the centre of the plastic lid (from now referred as *sprayed dish*). A similar set up was also designed with 9 cover slips, producing more reliable data for the relation of the spore deposit to the distance from centre of the sprayed dish (Figure 3.5 -*B*). 5 replications were conducted for both 5, and 9-cover slip experiments.

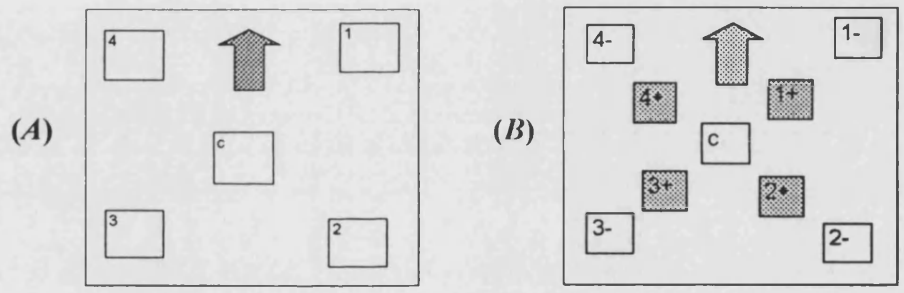


Figure 3.5 Set up for testing the homogeneity of the spray deposit of the Potter tower.

A) Repli dish lid set up with five cover slips, 4 on the corners and 1 in the centre;

B) Set up with 9 cover slips, 4 on the corners (outer zone) (-), 4 in the inner zone (+) and 1 in the centre.

The cover slips on the dish, were sprayed with 1.5ml suspension of Uvitex BHT stained spores, of concentration 1×10^6sp/ml. The spores were left to dry, and were observed under an Olympus BH2 microscope. 5 counts were done, on different viewing areas, which were randomly selected on each cover slip. The microscope viewing field at magnification 200× was calculated as 0.478 mm^2. All the spores on a viewing field were considered as one count.

Investigating the relation between dose applied and spore deposit

In these experiments, the relation between the spore concentration and the spore deposit was investigated. 1.5 ml of (Uvitex BHT stained) spore suspensions, with concentrations ranging from $3x10^5$ to $3x10^7$sp/ml, were applied on aphids, and on glass cover slips. The aphids were placed on a 5cm Petri dish, with an appropriately cut pepper leaf embedded on water agar. Both aphids and cover slips were placed in the centre of the spray disk when sprayed, and afterwards were left to dry for a few hours. The aphids were killed by freezing (-20°C).

For observations, an Olympus BH2 (UV) microscope was used at 200× magnification. The number of spores on a viewing area was counted for both aphids and cover slips (viewing area=0.478 mm^2). It was impossible to count the total number of spores on the whole aphid body, with high accuracy. The main reason was that the curved body of the aphid, did not allow a universal focal distance. For that reason, the abdomen of the aphid was selected, as a fixed viewing area, and the spores were eventually counted by altering the focus distance (scrolling up and down as in Figure 3.6).

In this way, counting error was minimised, despite the complexity of the body surface. The final number of spores on an aphid was an estimate of the number of spores on the respective total aphid surface (*spore density* (sp/mm^2) × *aphid body surface* (mm^2)).

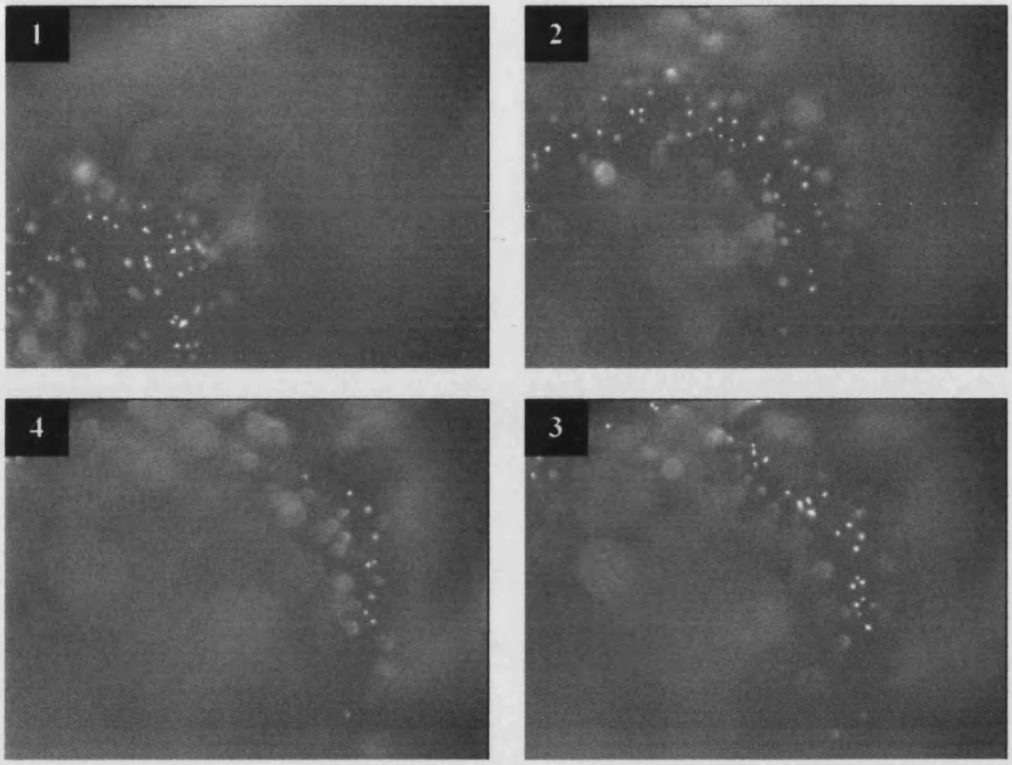


Figure 3.6 The view of the dorsal side of an aphid sprayed with Uvitex BHT stained spores at different focus distances using Olympus BH2 (UV) microscope.

The viewing area remains the same in all 4 frames. The distance from the object (aphid) is increased from frames 1 to 4. The spores in focus can be spotted only in a narrow area in each frame while the rest of the image is bleary. Dose applied 10^7sp/ml.

Effects of Triton-X 100 on the homogeneity of the spray deposit

This experiment was designed to investigate whether the use of 0.02% Triton-X 100 in the applied suspension, reduced the variance of the spore counting results, that was observed in previous experiments. The '5-cover slip' experiment was repeated using a 10^6sp/ml suspension.

3.2.2.3 *V. lecanii* spore germination

The germination of *V. lecanii* spores on growth media and on aphids was investigated in the following experiments.

Assessing germination on BPA media

Germination of *V. lecanii* spores was determined using the method of Hall (1977). The spores were germinating on a thin layer of bacteria peptone agar medium (BPA), on a glass slide, where very accurate microscopy observations could be made. Under a laminar flow cabinet two drops of melted sterile BPA was placed on a sterile glass slide, creating two small media pools. Once the BPA was set, 3 slides were placed on bent glass rods, in sterile repli dishes (no compartments) (Figure 3.7). The glass rods were sitting on three layers of sterile filter paper (Whatman No 3), moistened with sterile distilled water, to maintain a saturated environment in the dish. Spore suspensions from 5 different *V. lecanii* colonies were prepared, with spore concentrations around 10^6sp/ml (method described in Chapter 2). One drop of 0.01 ml of spore suspension was placed on one BPA pool, from each *V. lecanii* colony, and left to dry for 5 minutes. The repli dish was then sealed with parafilm M®, and was incubated in dark at 24°C. Germination was assessed at 6, 8, 10 and 12 hours after inoculation. For each interval, one repli dish was prepared and the spores were killed and visualised using 0.1% cotton blue in lactophenol. The spores' germination was observed under a light microscope. A spore was considered to be germinated, when the germ tube, was greater or equal, to half the length of the spore. The spore germination was estimated in 5 randomly selected viewing areas, for each BPA pool.

Assessing germination on aphids using UV microscopy

10 adult aphids were placed on a 25 square-compartments repli dish, with a cut-to-fit pepper leaf, embedded on water agar, in each compartment. The aphids were sprayed with known concentration spore suspension of Uvitex BHT stained spores, and were allowed to dry for 5 min. The repli dish was sealed with parafilm M®, and ventilation holes were made over each compartment to allow gas exchange. The germination on the aphids was assessed under UV microscope (Figure 3.8), after specified time interval. Preliminary experiments showed great variation in results. Control of the following factors resulted in consistency among the observations.

Age: All the aphids were 1-2 days old adults.

Honey dew residues: The aphids tested, were reared and incubated in individual chambers, to avoid honeydew contamination of their cuticle from neighbouring insects' excretions.

Humidity: The repli dish was placed in sealed transparent box with distilled water moistened filter paper, to ensure homogenous and high relative humidity.

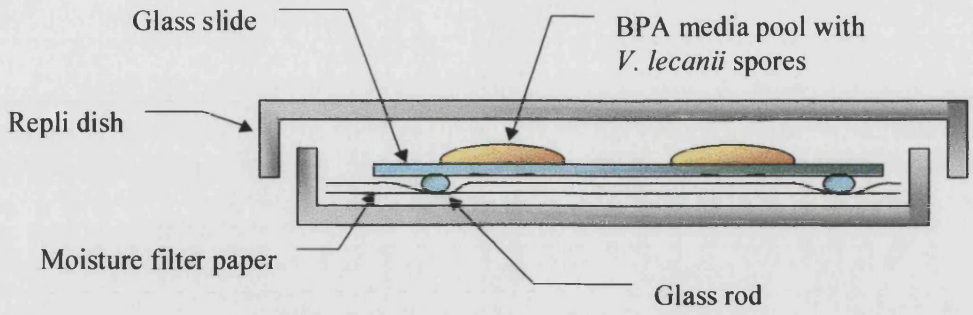


Figure 3.7 The repli dish set up for estimating spore germination on a BPA pool.

The glass slide was placed on the bent glass rods. The repli dish was sealed with parafilm M®. The moist filter paper kept the environment saturated. The spores germinated on the BPA pool.

Figure 3.8 Uvitex BHT stained *V. lecanii* spores germinating on the dorsal side of the thorax of an adult aphid after 24 hours of incubation (200×).

Assessing germination on aphids using Cryo. SEM

10 adult aphids were prepared as described in the previous experiment. Aphids were killed using chloroform under a fume cupboard. The Cryo. SEM system used was a Jeol JSM6310 (Jeol, Tokyo, Japan). The 4 aphids were placed on the specimen holder, and were plunged into nitrogen slush, rapidly to freeze these specimens. After a few minutes the specimens were transferred to the SEM chamber, while the system was kept cold with liquid nitrogen (-185°C). Each specimen was then examined at low KV, to determine whether detail was obscured by frost. Then, the system was set at -85°C, for sublimation of the ice from the surface of the sample at a controllable rate. When sufficient ice had been removed, the specimen was transferred from the SEM chamber to the cryo. prep. stage. Sputter coating was commenced at -170°C for 2-3 minutes. The sample was again transferred to the SEM cold stage chamber and was observed at 10 to 5 KV.

3.2.2.4 Effect of Triton X-100 on the efficacy *V. lecanii* applications

To study the effects of the presence in the spore suspension of the wetting agent Triton X-100, on the efficacy of *V. lecanii*, an *'in situ'* dose response experiment was carried out, with 0.02% Triton X-100, or plain water suspension, both from the same spore patch (see Chapter 2). The experiment was repeated 3 times for concentrations of 10^3, 10^4, 10^5 and 10^6sp/ml. Mortality was recorded after a 7-day incubation.

3.2.2.5 Spore deposition on the aphid body using different application methods

In these experiments, an attempt was made to find and quantify spores on aphids, after different application protocols. 'Direct impact', and 'secondary pick up' methods were tested, using Uvitex BHT stained spores. Spore concentration $5x10^6$sp/ml was used for 'direct impact' trial. For 'secondary pick up' trials concentrations ranging from 10^6 to 10^8sp/ml were tested. The aphids, after being killed by freezing, (-20°C, overnight), were observed under an Olympus BH2 microscope.

3.2.2.6 Preliminary 'secondary pick up' experiments

The following experiments are all variations of the 'secondary pick up' experiment described in Chapter 2.

Determining the minimal exposure interval

This experiment was designed, to determine an optimum exposure interval of aphids to conidia infected leaves, for the 'secondary pick up' experiment. Four replicates (25 insects each) were exposed to leaves infected with 10^6sp/ml-spore suspension. The exposure intervals were at 0, 6, 12 hours, and then, at every 24h up to 168h (7 days). Aphids were then transferred to clean individual chambers in a repli dish, preventing further exposure to the fungal spores. Mortality was assessed 7 days from the time that the aphids were first exposed to the infected leaves.

Assessing the the spore viability on a leaf surface

The effect of time on the viability of spores, applied on a leaf, was investigated in this experiment. Pepper leaf disks were embedded on water agar (1% w/v), in a 9cm Petri dish. The leaves were sprayed with 1.5 ml 10^4sp/ml *V. lecanii* spore suspension, and 1.5 ml of sterile dH_2O, for control plates. The leaves were allowed to dry for 15 min, and then, the dishes were partially sealed with Parafilm M®. The dishes were incubated for 0 to 7 days, at a temperature of 24°C and photoperiod of 16h L: 8h D.

At set times, a dish was removed from the incubator, the sprayed leaf was eased off the water agar, and was gently pressed down on the surface of an antibiotic Malt agar Petri dish; (2% Malt Extract, 2% Agar (w/v), cyclohexamide 0.5g/l (in water) and chloramphenicol 0.4g/l (in ethanol)). The dish was then incubated for 4-6 days (24°C, dark), until fungal colonies were clearly visible. The viability of the spores on the leaf surface was estimated by the number of the colony forming units (*c.f.u.*), in relation to the dose applied.

Assessing the effects of spore viability on aphid mortality

The effect of time on the viability of spores applied on a leaf surface, and the subsequent effects on mortality were investigated, in this experiment. Leaves were sprayed with 10^8 and 10^9sp/ml spore suspensions. Aphids were introduced to the leaves 0 - 7 days after application, and were exposed for 24 hours to the fungal spores. The experiment continued as a typical 'secondary pick up' experiment (see Chapter 2). The mortality was assessed 7 days from the day that the insects were first introduced to the infected leaves.

3.2.3 Retrospective analysis of data- Effect of variability in spray deposits on mortality in bioassays

The dependence of spore deposit, on the distance from the centre of the spray disk, could have an effect on the mortality recorded in bioassays. In a set up like the *in situ* bioassay, an aphid will remain in the cell through out the spraying and incubation period. The dose that the insect will be exposed to, will depend on its position in the repli dish grid. Insects in the outer zone could receive less than half as many spores as insects in the inner zone although the same spore suspension was applied (Figure 3.9).

In experiments like 'secondary pick up' and 'direct impact', the position of the aphid in the repli dish, has nothing to do with the spore dose received. However, other factors could interfere with the mortality recorded in different zones in the Petri dish. Cells near the edge of the dish could have better ventilation compared to inner zone cells; they are close to the dry air environment, and the parafilm® seal with the Petri dish was not perfect creating additional ventilation (Figure 3.10). This could reduce the RH in the cell, affecting the infection development.

Data from 50 experimental trials were reviewed in this new way. The experiments were split in 'cell position - spore deposit' dependent, and independent experiments. The data collected were split in outer and inner zone mortality. This way, both the effect of the dose variation and the effect of position, were investigated.

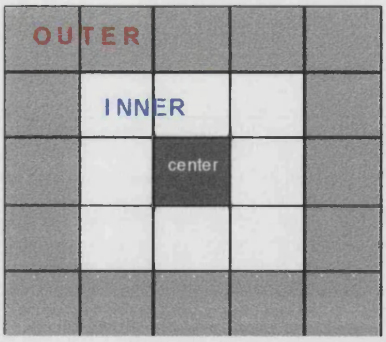


Figure 3.9 Zones on a bioassay repli-dish.

The aphid mortality in the two zones (outer and inner), was compared. In '*in situ*' experiments, the dose received by the aphids, dependent on their position on the repli dish grid (same spore suspension applied but different spore deposits). In 'secondary pick up', and 'direct impact', the spore dose was independent to the position.

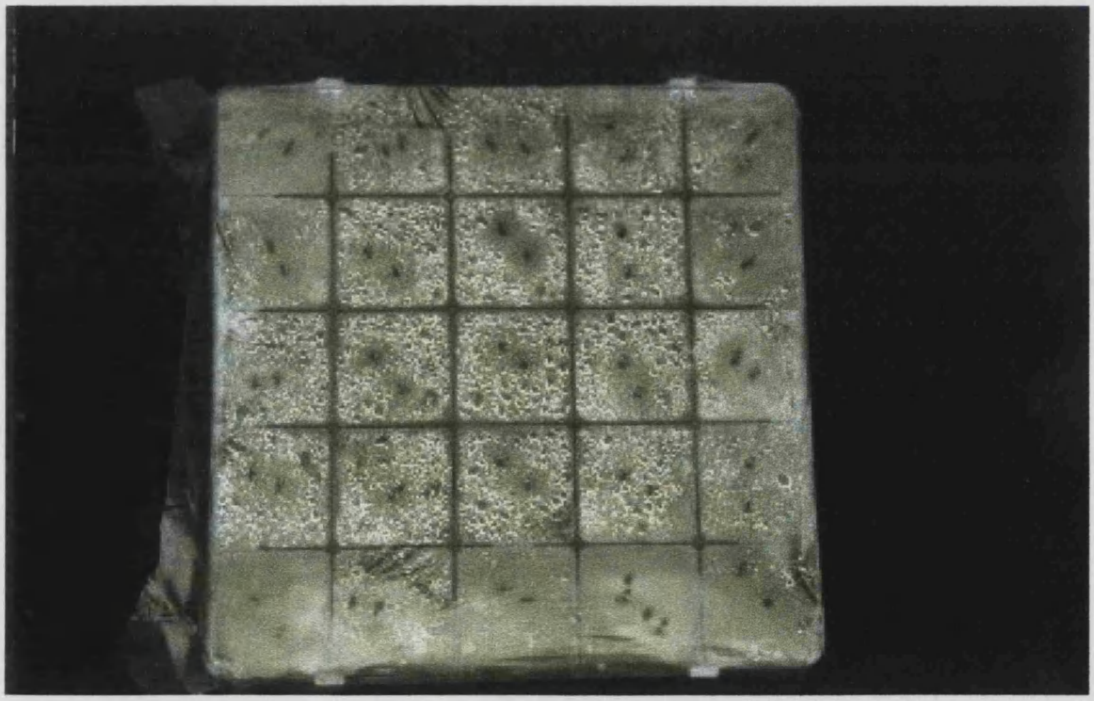


Figure 3.10 Petri dish covered with parafilm M® after 4 days of incubation.

Droplets of water are formed on the parafilm M® surface, creating almost a humidity-saturated environment. Ventilation holes over each camber are visible, allowing gas exchange. The number of water droplets is higher in the inner zone compared to the outer zone. despite the fact that the parafilm is perfectly fitted from all sides.

3.2.4 Methods of spore pick up comparative experiments

3.2.4.1 *In situ*

In situ dose response experiments were carried out with all 3 insecticide resistant (R1, R2 and R3) and susceptible (S) clones (see Chapter 2). The doses applied ranged from 10^3 to 10^6sp/ml. 50 insects were used per treatment. The mortality was assessed after a 7-day incubation period.

3.2.4.2 Secondary pickup

Typical experiment

All four *M. persicae* clones were tested in 'secondary pick up' dose response experiments (see Chapter 2). The applied doses ranged from 10^5 to 10^8sp/ml. 50 insects were used per treatment. The mortality was assessed after a 7-day incubation period.

7-day exposure

All four *M. persicae* clones were tested in 7-day exposure (through out the experiment) 'secondary pick up' experiments. The dose used was 10^6sp/ml. 50 insects were used per treatment. The mortality was assessed after a 7-day incubation period.

3.2.4.3 'Direct impact'

Clones R1 and R3 were tested in comparative 'direct impact' dose response experiments (see Chapter 2). The applied doses ranged from 10^5 to 10^8sp/ml. 50 insects were used per treatment. The mortality was assessed after a 7-day incubation period.

3.2.5 Method of horizontal spore transmission experiment

This experiment was designed to investigate the horizontal spore transmission from an infested aphid, to neighbouring insects. The ability of the mycosed insect to infect was tested at different levels of infection development. *M. persicae* clone R1 was used for this experiment.

The infecting aphids (source of inoculum) were sprayed with a spore suspension of 10^6sp/ml. The aphids were then incubated (conditions: 24 °C, 16h L: 8h D) for 7 days (infection at maximum sporulating level), 3 days (beginning of sporulation) or 1 day (germinating *V. lecanii* spores), before they were placed together with the healthy individuals. One infected aphid and 2 untreated adults were placed together in a small chamber of 2×2 cm of a repli-dish. Three exposure intervals were tested; 1, 2 or 3 days. The healthy aphids were then transferred to uninfected individual chambers. Mortality was assessed after 7 days of incubation. All repli-dishes were prepared as described in Chapter 2.

Mycosed aphids could easily be distinguished from the untreated, present in the same chamber. Treated and untreated insects could also be distinguished when the 'infected

aphids' were incubated for 3 days, because their bodies were slightly darker and pink in colour compared to the bodies of untreated healthy aphids. But 'infecting aphids', incubated for just one day, could not be distinguished from the untreated adults, unless marked with non-toxic paint. A small black dot (watercolour) was painted on the back of the aphids with a fine brush (size: 3 zero). The watercolour neither effected the aphids, nor subsequent fungal growth and sporulation, as was shown in preliminary bioassay trial (Figure 3.11).

Figure 3.11 Sporulating adult aphid marked with black water colour on the back of the abdomen.

3.3 Results

3.3.1 Results of preliminary experiments

The current set up was first used by Williams (1995) who experimented on the gas pressure during application, and the effects of the increasing sprayed volume, as well as the increase of the suspension concentration, on the spore deposition. A good spectrum of droplet size and even coverage was achieved, with nozzle pressure at 10psi. The recommended application rate for *V. lecanii* by Koppert BV was 1500 - 3000 l ha^{-1} of 10^6sp/ml. Williams (1995) estimated that 1.5ml of the suspension applied with the potter tower would give a similar coverage rate.

The first aim of the preliminary experiments was to establish a relationship between concentration of the applied suspension and the spore deposit on aphids. In order to associate the deposit (sp/mm^2) to the total number of spores hitting the target, (sp/aphid) a 3-dimentional model of an average size aphid was developed. The homogeneity of the spray deposit, in relation to the distance from the centre of the spray disk, is also tested.

3.3.1.1 Estimating the body surface of an aphid

Image analysis

The surfaces of 15 aphids were estimated from microscope images with the programme Visilog™ (Noesis). The resulting value was 0.92 ± 0.30 mm^2. The total aphid surface according to this result is (0.92×2) = 1.84 mm^2.

3D Model

The results from measuring body dimensions of 10 adult aphids are shown in Table 3.1.

Table 3.1 Average adult aphid body dimensions in (mm).

	Body length (h)	*Width (b)*	*Eye distance (a)*
Mean	1.43	0.70	0.34
SEM	0.04	0.02	0.01

The dimensions required by the 3D model were estimated under a light microscope from 10 adult aphids. *SEM* for standard error of the mean.

The model was applied to all ten adults and the mean aphid body surface estimate was 2.35 ± 0.10 mm^2 (*mean and SEM*).

3.3.1.2 Calibrating the Potter tower- Homogeneity of the spray deposit

In both experiments (5 or 9 cover slip trial) 5 replicates were made. On each cover slip, 5 random viewing areas were selected, and the spores were counted under a light microscope. The results for the '5-cover slip' experiment are displayed in Figure 3.12 (see Figure 3.5-*A* for experiment design). The spores counted per viewing area, (0.478 mm^2 for magnification 200×) on the covers slips, on the corners of the dish ranged from 16.0 to 11.5, while on the cover slip on the centre, an average of 35.3 spores/'area of view' was recorded. Statistical analysis revealed significant difference in the spore deposit between the corners and the centre of the sprayed dish. However, there were no differences between the corners.

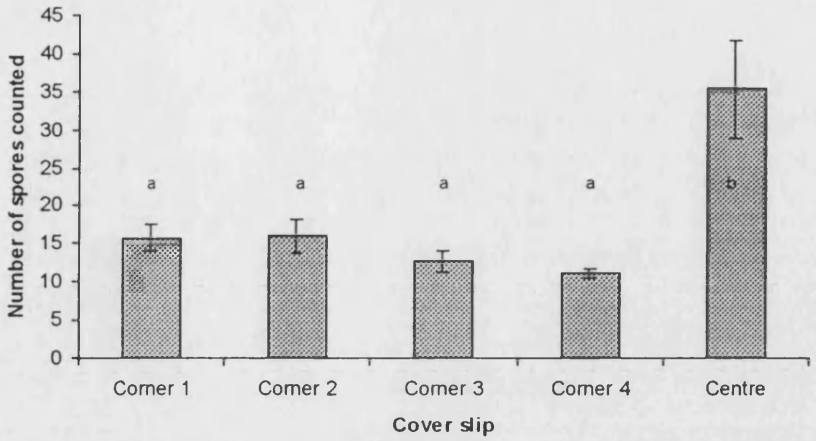


Figure 3.12 Spores counted at different sides of the sprayed disk in a 5-cover slips experiment.

The cover slips at corners of the sprayed dish are marked with numbers. The columns represent the arithmetic mean (spores counted in the viewing area) of five counts of five covers slips per position. The error bars represent standard error of the mean (SEM) (n=25). *ANOVA* test was applied for comparison between the position. Columns with different letters are significantly different ($P<0.05$).

In the 9-cover slip experiment, the interest was focused on the differences on the spore deposits in relation to the distance from the centre of the sprayed dish (see Figure 3.5-*B* for experimental design). The results are displayed in Figure 3.13. The average spores counted in the outer zone was 15 per area of view (0.478 mm^2 for magnification 20×10), and for inner and central was 21 and 28 respectively. Statistical analysis showed that spore deposits differ significantly in relation to the distance from the centre of the sprayed dish.

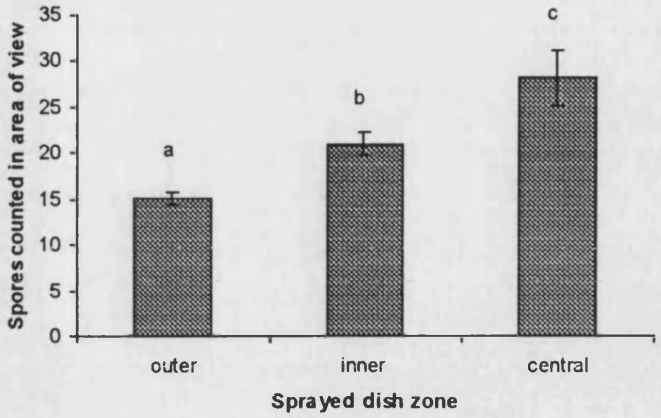


Figure 3.13 Spores counted in different zones of the sprayed dish in a 9-cover slip experiment.

The cover slips are grouped in outer, inner and central zone. The columns represent the mean (spores counted in the viewing area) of 5 counts of 20 covers slips per zone (5 cover slips for the central zone) and the error bars for SEM (n=100, 25). *AVOVA* test was applied for comparison between the zones. Columns with different letters are significantly different ($P<0.05$).

3.3.1.3 Calibrating the Potter tower- Relation between dose applied and spore deposit

Adult aphids and cover slips were sprayed with a range of concentration of Uvitex BHT stained spores. The aphids were observed both on the ventral and dorsal side of their bodies. The spore counting results are shown in Table 3.2. For each count a viewing area of 0.487mm^2 was used under 200× magnification.

Table 3.2 Spores counted on glass cover slips and on aphids (dorsal and ventral side) for different spore suspension concentrations.

	Spore concentration (sp/ml)					
	$3x10^5$		$3x10^6$		$3x10^7$	
	Mean	SD	Mean	SD	Mean	SD
on glass cover slips	7.4	5.4	97.4	48.5	828.3	442.9
on Aphids (Dorsal)	1.3	2.8	70.3	31.5	418.6 *	1.0
on Aphids (Ventral)	0.8	1.1	20.5	26.9	90.0	116.4

The mean and the standard deviation (SD, n=15 on cover slips, n=5 on aphids) are displayed. The numbers represent sp/mm^2. Numbers followed by (*) represent estimated data due to the inability to accurately count the observed spores.

The number of spores counted on the glass covers was always higher from the other two surfaces, for all concentrations applied. The lower number of spores was always found on the ventral side of the aphids. However, in all cases high standard deviations were observed. Observing a treated surface just after application, it is possible to see formation of spore suspension droplets (Figure 3.14-A). In general, the application is homogenous, but under the microscope, areas with lower spore deposits can be observed (Figure 3.14-B). This phenomenon accounts for the observed variance.

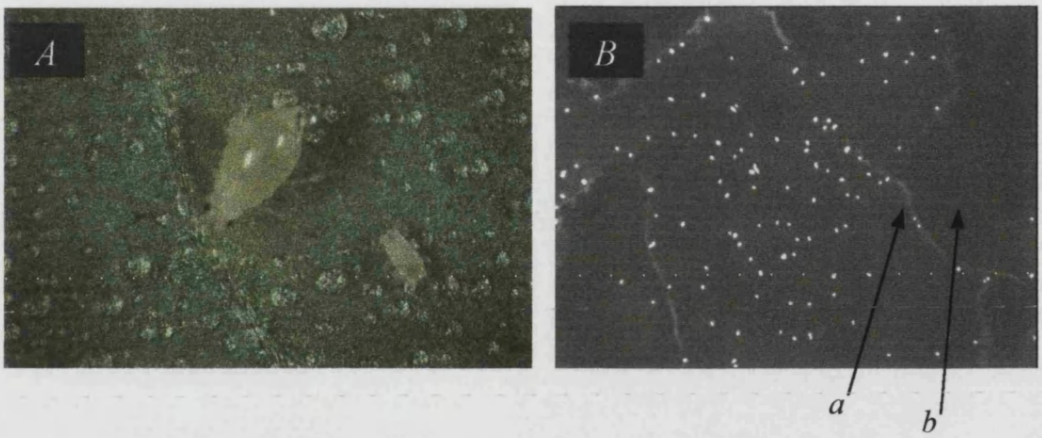


Figure 3.14 (*A*) A leaf just sprayed with 1.5 ml of water spore suspension and (*B*) Sprayed cover slip with Uvitex BHT stained spores under a UV microscope (200x)

(A) The droplets are visible on the leaf surface.

(B) The limits of the suspension droplets on the sprayed cover slip are also visible under the microscope (pointing arrow *a*). Areas outside the limits of the droplets have low spore deposits (arrow *b*). This creates high variation among the results recorded when counting spores on randomly selected viewing positions. (suspension applied: 10^7sp/ml)

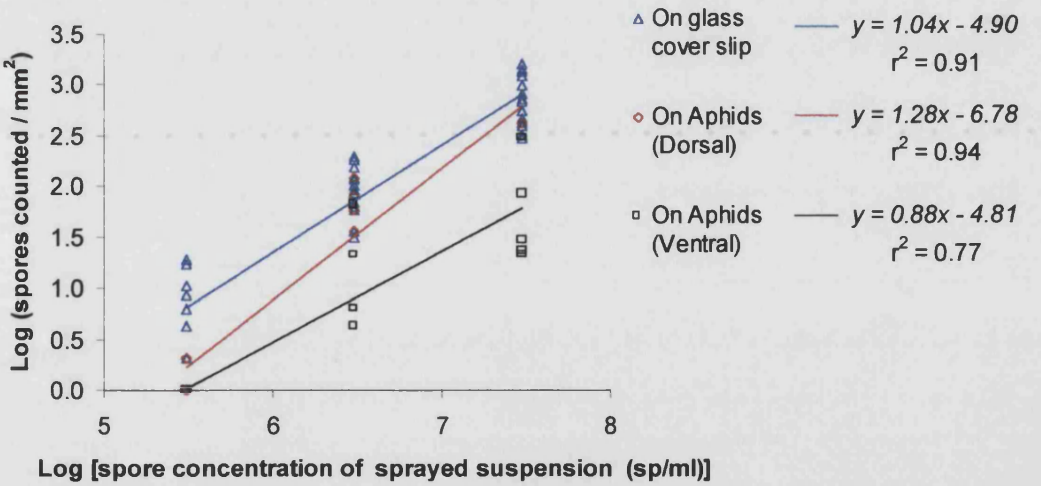


Figure 3.15 Relation between spores applied and spores deposited when using the Potter tower.

Spores deposited on glass cover slips and aphids (dorsal and ventral side) for applied concentrations 3×10^5, 3×10^6 and 3×10^7sp/ml. The data were fitted on a linear model (r^2 for coefficient of determination).

The spore counting results were plotted on logarithmic scales and fitted to simple linear regression. The data from all the surfaces showed a significant positive correlation (from the *product moment correlation coefficient (r)*, $P<0.05$; $df=43$, on cover slips, $df=13$, on aphids). The data from the cover slip and the dorsal side of an aphid fitted a best-fit linear model well ($r^2>0.90$). The slope of the linear equation was around 1 for both treatments. Consequently, there was a direct relation between the applied dose and the spore deposit for these treatments. The data, from the spore deposit on the ventral side of the aphids, did not fit the linear model as well as the previous data sets. About 27% of the spore pick up was not accounted for the increase of the applied spore concentration.

From the equations (Figure 3.15), it is possible to estimate the concentration of spores deposited on the sprayed surface or on aphids from the spore suspension applied.

3.3.1.4 Effects of Triton X-100 on the homogeneity of the spray deposit

Spore counting was considered to be inaccurate because the presence of the wetting agent Triton X-100 allowed the formation of massive spore clumps. The clumped spores could not be counted. The spores furthermore, were not evenly distributed, and estimating the spore deposits on the cover slip was meaningless. The variance of the spore counting results on cover slips was not reduced using Triton X-100.

3.3.1.5 *V. lecanii* spore germination on BPA

Spore germination of *V. lecanii* conidia was extensively studied by Hall (1977). He found that optimum temperatures for germination ranged between 20 and 25 °C, and that there was a broad spectrum of optimum pH, from 4.5 to 8.5. Light and spore density had no effect on the resulting germination. The experimental set up was designed on the basis of these findings.

The spore germination was stopped with cotton blue in lactophenol, after a specific time interval, and was immediately assessed. The data are displayed in Figure 3.16. After 6h, a low 14% germination was observed, and reached 100% within a12h incubation. The median germination time[1] was estimated using probit analysis at 7.9h (0.1 (95% confidence limits)).

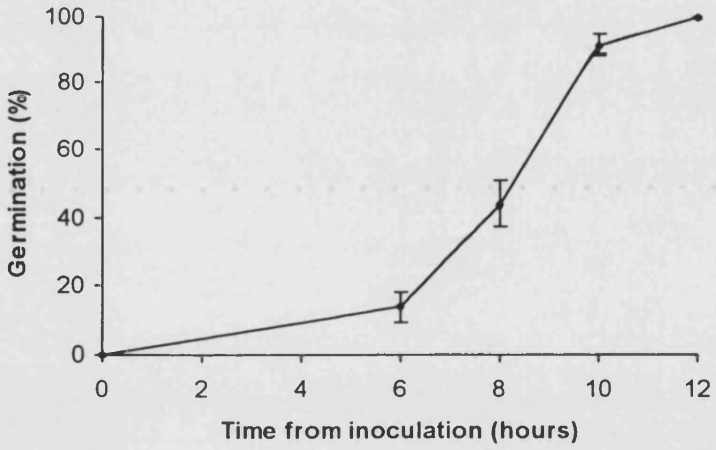


Figure 3.16 *V. lecanii* spore germination on Bacteria peptone agar (BPA media).

The germination % was assessed from 5 different spore suspension by counting the spores in the field of view of 5 randomly selected position on the media pool. The points represent the arithmetic mean and the error bars standard deviation (n=25).

[1] Time interval required for half of the spores (50%) to germinate

3.3.1.6 *V. lecanii* spore germination on aphids

Preliminary experiments showed increased variation in observed spore germination. A case of extreme saprophytic surface growth is displayed in Figure 3.17. The modified method was used to minimise variation.

The data were collected from 9 aphids (24-h incubation) under UV microscope (200x). The median germination (%), on the dorsal and ventral sides of the aphids are displayed in Figure 3.18. 90% germination was recorded on the dorsal aphid side, and the interquartile region (C1-C3) indicates a narrow spread of the data. On the ventral side of the aphid lower germination was recorded (68%) with a wider data spread indicating increased variability of the results for this side of the aphid body.

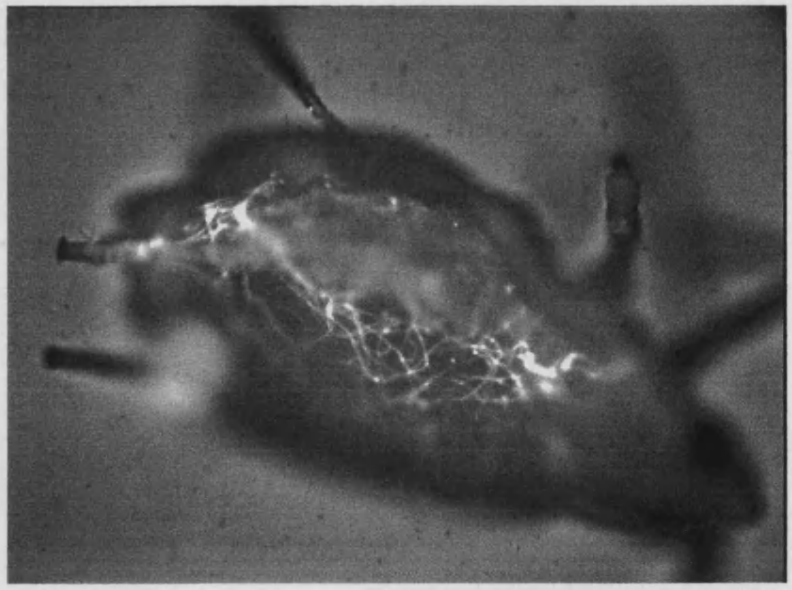


Figure 3.17 Saprophytic fungal growth on aphid cuticle after 48 h of incubation.

Stained fungal hyphae appear as a bright complex web on the aphid surface.

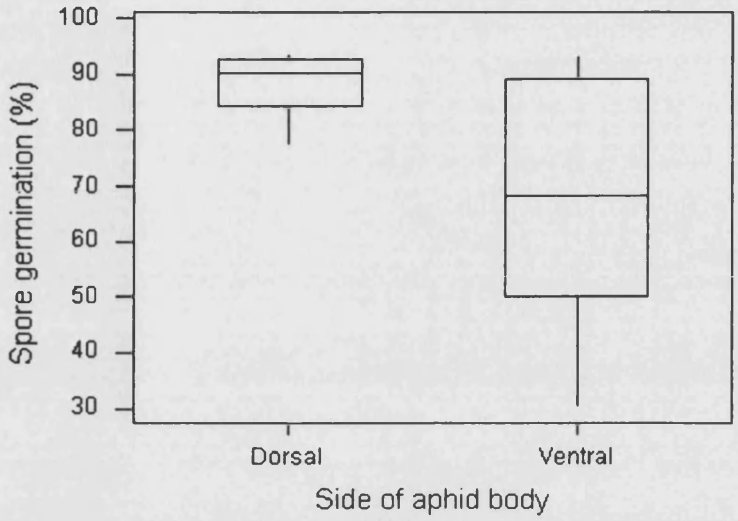


Figure 3.18 Spore germination on aphids after 24 h, using the improved method design.

The line within the box represents the median (n=9). The limits of the box represent the upper and lower quartiles (C1 and C4). Applied suspension: 4×10^6sp/ml.

Estimating germination using Cryo. SEM

The aphids were coated with gold, and were placed under the electron beam, after 24h incubation. Estimation of the germinated spores was done visually (Figure 3.19). However, the method had a few problems. It was possible to count spores only in a relatively restricted area, away from the 'glowing' edges of the aphid. The position of the aphid was fixed, and it was not possible to observe the ventral side. Accurate observation required high magnification, that slowed down spore counting.

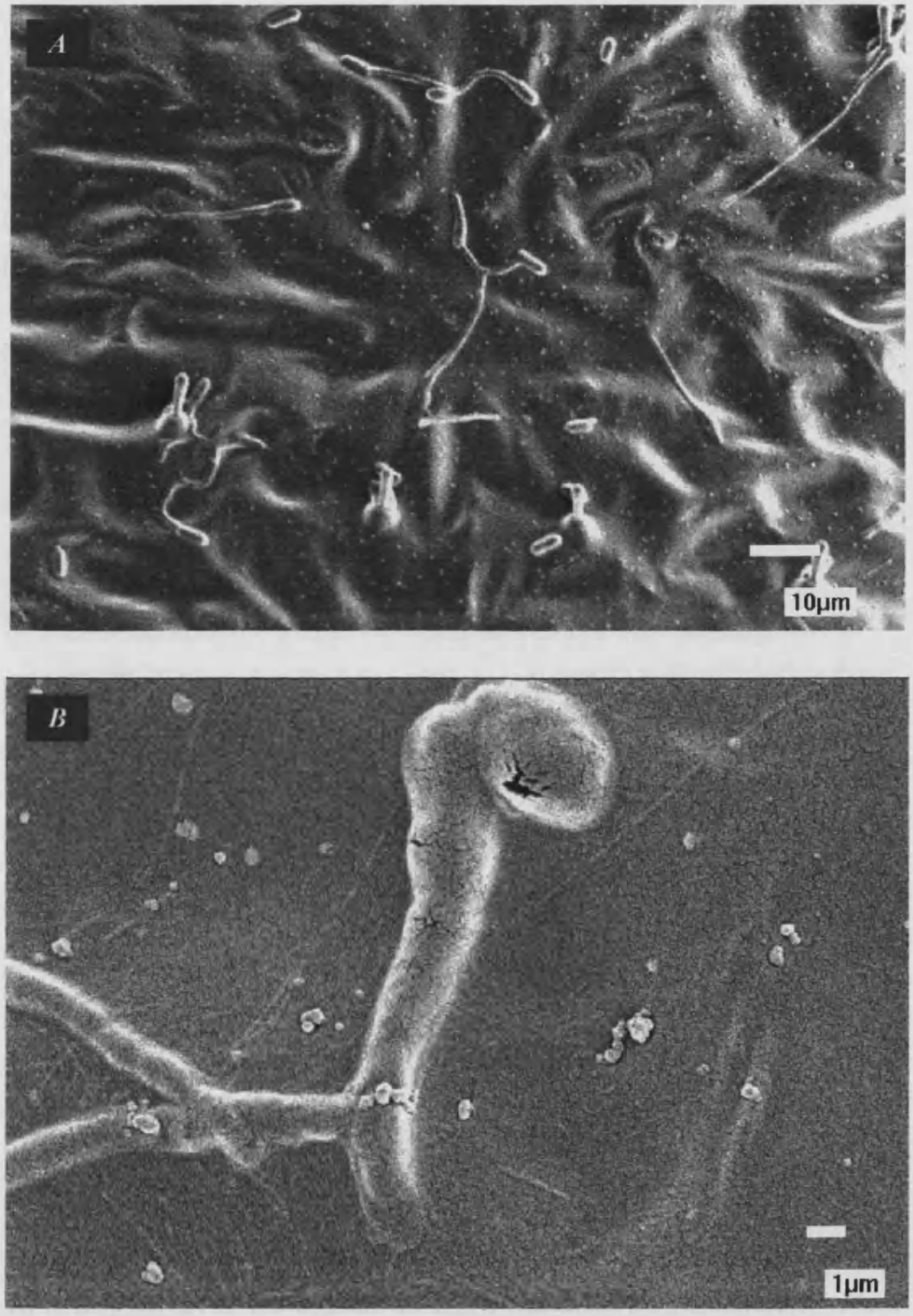


Figure 3.19 Spores germinating on aphid surface after a 24-hour incubation as displayed on the Cryo. SEM system screen.

(*A*) Typical screen used for spore counting, (*B*) Germ tube penetrating the aphid cuticle.

3.3.1.7 Effects of Triton X-100 on the efficacy of *V. lecanii* application

The dose response bioassay was repeated 3 times (total 75 insects per dose) with plain water or 0.02% Triton X-100. The total mortality (%) recorded on the last observation day of the bioassays is displayed in Figure 3.20. 70% mortality was recorded for both treatments, for a 10^6sp/ml applied dose. For dose 10^3sp/ml, 7% mortality was recorded for Triton X-100 treatment, and 1% for Water. Low control mortality was also recorded, 1.3% for 'Water' and 2.5% for 'Triton-X'. The dose response for the two treatments was estimated using the probitan program on Genstat. The data fitted a single linear model, showing no significant deviance (*df* =7), suggesting that the two treatments were not significantly different (P<0.05).

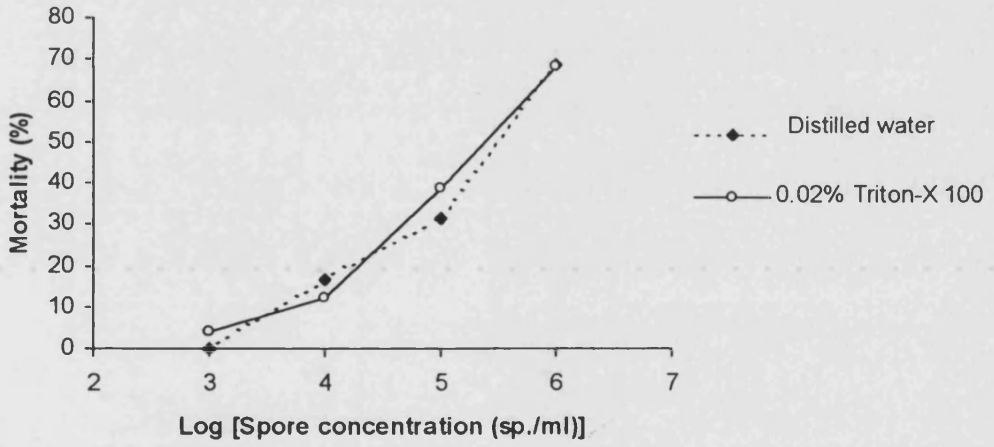


Figure 3.20 Mortality (%) recorded on day 7 of the dose response bioassay for treatments Water and 0.02% Triton X-100.

The application method *'in situ'* was used for the bioassay. The experiment was repeated three times (25 insects for each rep., per dose). The points on the graph are the total mortality from all three experiments corrected for control mortality with Abbot's formula (see Chapter 2). The probitan program for calculating the LC_{50} was used for statistical analysis. No significant difference was found between the treatments (P<0.05).

3.3.1.8 Spore deposition on the aphid body using 'direct impact' application method

The sprayed insects were killed by freezing, and the spores were counted on their dorsal and ventral sides under a UV microscope. The results are displayed in Figure 3.21. On average 60 spores were found on the dorsal side and 9 on ventral side. The observed difference was significant ($P<0.05$). Using the 'direct impact' application method, 88% of the spores was located on the dorsal side of the insects, which was also the side exposed to the spore suspension.

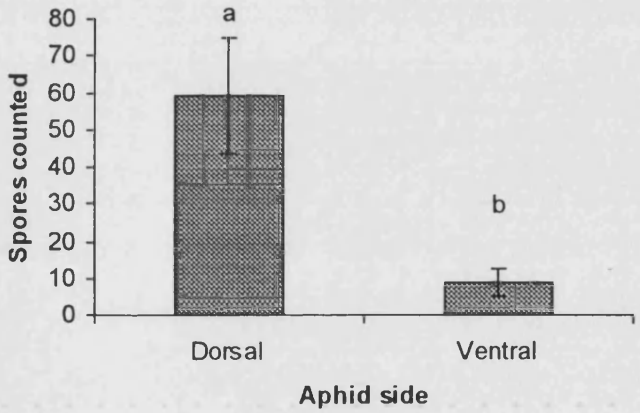


Figure 3.21 Spores counted on the dorsal or the ventral side of an aphid sprayed with 'direct impact' application method.

Columns represent arithmetic mean and error bars standard deviation (n=5). Mann Whitney *U* test was applied on the data for comparison between the two sides. Columns with the same letter are significantly different ($P<0.05$). Suspension applied 5×10^6sp/ml.

3.3.1.9 Spore allocation on the aphid body using 'secondary spore pick up' method

Spore concentrations of 10^6 and 10^8sp/ml were tested. In the first case, the number of spores counted was very low. The spores were mainly found on the legs or on the mouthparts. Spores on the abdomen were almost absent. For spore concentration 10^8sp/ml, spores again were mainly found on the legs, but also patches of spores were present on the abdomen, and the thorax on the ventral body side (Figure 3.22). Great variation in the number of spores counted was observed (12 - 180 spores counted per adult). The results are not presented for this reason. However, this is an indication of the accidental way that the spores are picked up during the exposure interval. In some cases, spores in patches were found on dorsal side, probably picked up when aphids rolled over.

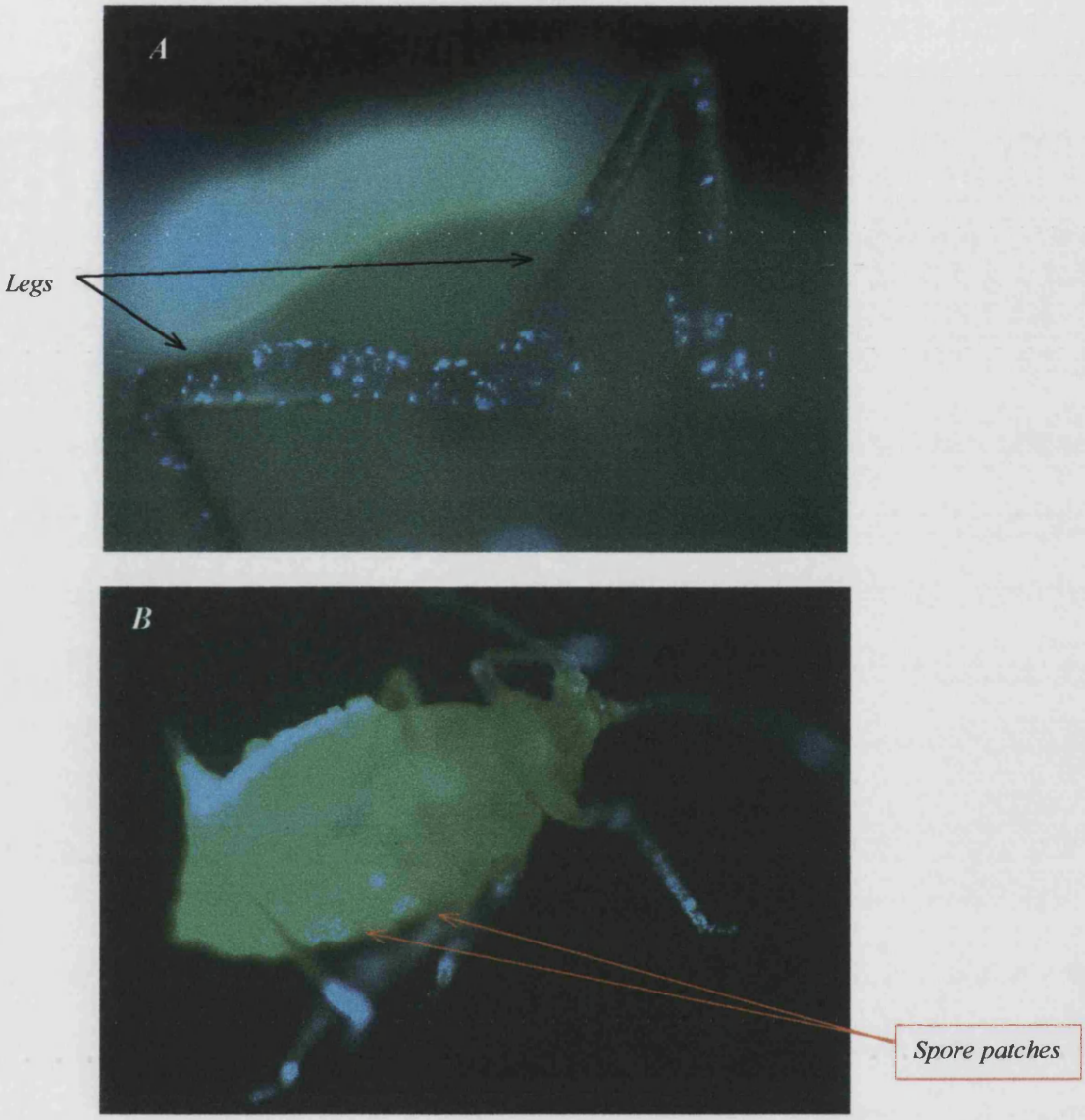


Figure 3.22 Location of *V. lecanii* spores on the aphid body in secondary pick up experiments using Uvitex BHT stained spores.

The spores were mainly located on the legs (*A*) or on the ventral side of the abdomen usually in patches (*B*). Images are taken under a UV microscope, 200× for image (*A*) and 100× for image (*B*). Applied dose 10^8sp/ml and 24h exposure to the infected leaf.

3.3.1.10 Effect of the exposure interval on secondary spore pick up

The aphids were exposed for different intervals to the sprayed leaves. The mortality recorded 7 days after the insects were first exposed to spores, is displayed in Figure 3.23. No control mortality was recorded (not displayed). Aphid mortality was first recorded after 12-h exposure (4%). Maximum mortality was recorded for 168-h exposure (50%). There was a significant positive correlation between exposure time and mortality ($r=0.970$, for $df=8$, $r_t=0.838$, $P<0.01$)

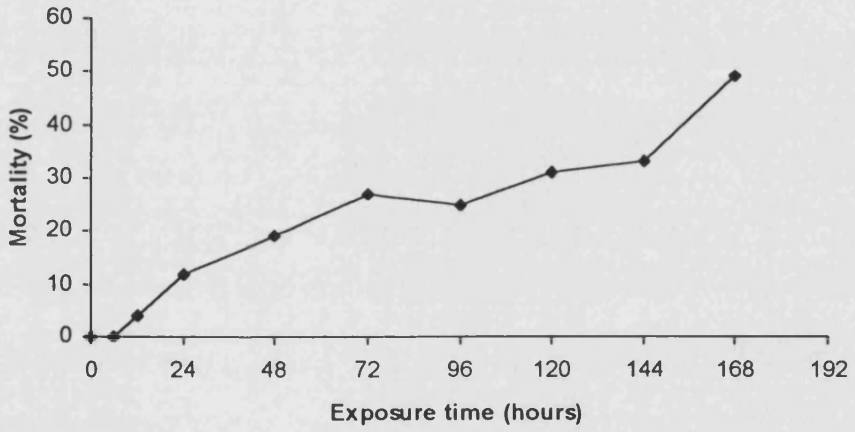


Figure 3.23 Mortality (%) recorded in 'secondary pick up' experiments after different exposure intervals to the infected leaf surface.

The aphids were applied on the infected leaves for 0, 6, 12, 24 and up to 168 hour (every 24 h). The mortality was recorded 7 days after the insects were first exposed to the spores. The points represent the over all mortality (4 replications, 25 insects each). Suspension applied; 10^6sp/ml. There was a significant positive correlation between exposure time and mortality (%) ($P<0.01$).

3.3.1.11 Effect of time on spore viability on leaves

In this experiment, spore viability was studied, in relation to the time interval that the spores were allowed on a leaf surface, after application. The colony forming units from leaf imprints on antibiotic Malt Agar after a time interval (0 to 7 days after application) were counted. The results from 3 replicates are displayed in Figure 3.24.

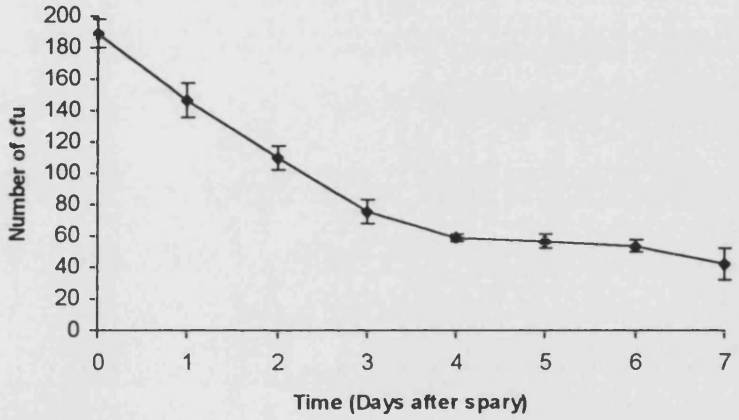


Figure 3.24 The effect of time on the spore viability on a leaf surface.

The spores were left on leaves for 0 -7 days after spraying. The number of colony forming units (*c.f.u.*) from the leaf imprint on a antibiotics-Malt agar medium are illustrated on this graph. The points represent the arithmetic mean (3 replications) and error bars the SEM. The results show a significant negative correlation between time and number of c.f.u. (r=-0.906, df=19, r_t=0.546 for $P<0.01$).

3.3.1.12 Effect of spore viability on secondary pick up

In this experiment the relation between the mortality recorded in 'secondary pick up' experiments and the time that the aphids were introduced to the sprayed surface after application was investigated. The selected time intervals ranged from 0 days (typical 'secondary pick up' set up) up to 7 days after application (applied doses: 10^8 and 10^9sp/ml). The aphids were introduced to the surface for 24h. The results are displayed in Figure 3.25. There was a significant negative correlation between the mortality and the time (r=-0.815 for 10^8 and r=-0.744 for 10^9sp/ml. df=19, r_t=0.549, P<0.01).

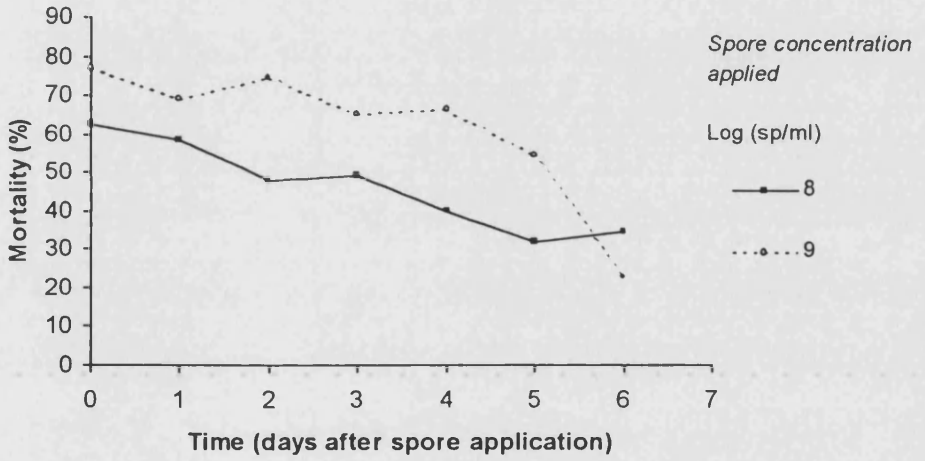


Figure 3.25 Mortality (%) recorded in 'secondary pick up' experiments when the aphids were introduced to the fungus-inoculated leaves after different time intervals from spore application.

Aphids were applied on the leaf surface at 0 - 7 days after conidia application. Aphid mortality (3 replications, 25 insects each) was recorded after 7day incubation. The points represent the over all mortality. The results show a significant negative correlation between mortality and time interval for both spore concentrations tested (P<0.01) Control mortality was zero (not displayed).

3.3.2 Retrospective analysis of data - Effect of variability in spray deposits on aphid mortality in bioassays

For each case examined ('spray dependent' and 'independent' bioassay set up) the mortality in 45 repli dishes was reassessed in zones as described in the Methods section. The results are displayed in Table 3.3.

Table 3.3 Mortality (%) of aphids recorded in the outer and the inner zone in 'spray dependent' and 'independent' experiments.

	Outer zone		*Inner zone*	
Spray Dependant	41.11	*a*	68.84	*b*
Spray Independent	44.03	*a*	50.32	*b*

In 50 repli dishes mortality was reassessed in inner and outer zones in experiments where the final spore deposit was related to the zone ('spray dependent') or not ('independent'). Numbers represent mean mortality (%) (n=50). Means within a row followed by the same later are not significantly different ($P<0.05$).

The data were analysed on Genstat assuming a binomial distribution. The program is displayed in Appendix 1. Significant differences were observed between inner and outer zones for both 'spray dependent' and 'independent' bioassay set-up. This suggests the following:

- The differences between the zones in 'spray independent' experiments indicates differences in the incubation conditions (the amount of spores deposited on the aphids was independent from the positions in the repli dish). Evidence that cells near the edge were better ventilated, compared to the cells in the centre, can be associated with the observed mortality differences (see Figure 3.10). The ventilation

of the edge cells probably resulted in a lower RH. This observation affects all repli dish bioassay experiments.

- The mortality differences observed between the zones in the 'spray dependent' experiments, were more significant ($P<0.001$) compared to spray 'independent' experiments ($P=0.024$). Previous findings (see section 3.3.1.2) indicated that cells in the centre of the dish would receive a significantly higher amount of spores in 'spray dependent' experiments. The combination of higher spore deposition with differences in RH resulted in an additive effect, increasing the mortality differences between the inner (central) and outer zones in the 'spray dependant' experiments.

3.3.3 Results of spore pick up comparative experiments

3.3.3.1 *In situ* experiment

This experiment was designed to compare the susceptibility of the insecticide resistant (R1, R2, R3) and susceptible (S) aphid clones to the fungus *V. lecanii*. The aphids were sprayed *in situ*, with concentrations ranging from 10^3 to 10^6sp/ml, and were incubated for 7 days. The results are displayed in Figure 3.26. The mortality (%) for all 4 clones showed a significant positive correlation with the applied dose ($P<0.05$). The highest mortality (70-80%) was recorded for a concentration of 10^6sp/ml. Low control mortality was observed for clones S and R2 (2-4%).

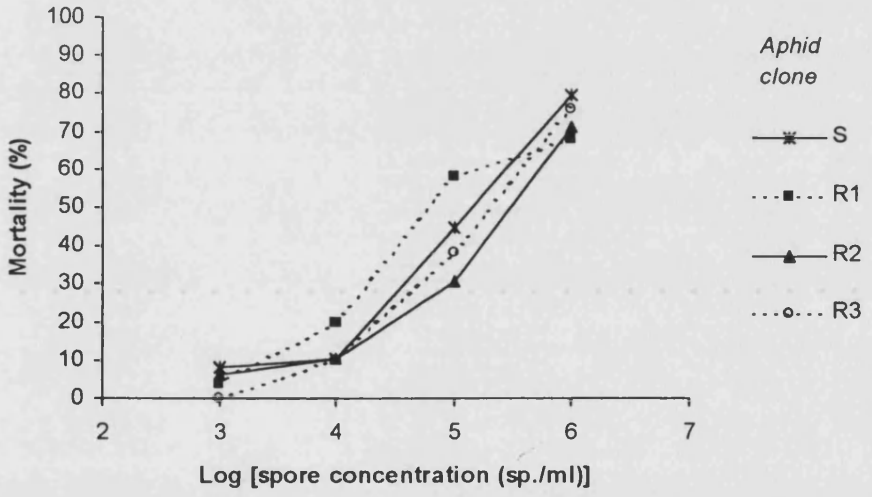


Figure 3.26 Aphid mortality (%) for clones S, R1, R2 and R3 sprayed *in situ*

In this dose response experiment the applied spore concentration ranged from 10^3 - 10^6sp/ml. After 7-day incubation interval the aphid mortality was recorded. The mortality (%) displayed was corrected for control mortality using Abbot's formula (see Chapter 2).

Table 3.4 Probit analysis of the dose response recorded for clones S, R1, R2 and R3 sprayed *in situ*.

Within clones

Clone	Zone	Log(LC_{50})	s.e.	*d.f.*	Deviance
S	*Inner*	4.43	0.24	7	8.47
	Outer	4.99	0.19	8	3.53
	All dish	4.78	0.15	7	0.95
R1	*Inner*	4.36	0.16	8	5.26
	Outer	5.47	0.20	8	5.03
	All dish	5.02	0.13	8	9.77
R2	*Inner*	4.64	0.24	7	5.65
	Outer	5.65	0.14	7	13.74
	All dish	5.31	0.14	7	6.66
R3	*Inner*	4.67	0.13	8	2.55
	Outer	5.59	0.17	8	6.39
	All dish	5.18	0.01	8	2.98

A

Between clones

Clones	Zone	Log(LC_{50})	s.e.	*d.f.*	Deviance
S, R1, R3 and R2	*Inner*	4.51	0.10	17	20.21
	Outer	5.43	0.09	17	26.09
	All dish	5.28	0.06	17	24.60

B

The LC_{50} was estimated separately for each zone and for the whole repli dish. The deviance was estimated using the single linear model that would indicated differences between the treatments. Significant deviance is followed by (*) for $P<0.05$ (*s.e.* for standard error).

(A) Differences within a clone: The variation between the trials (dose response experiments) for a clone was estimated. Significant deviance would indicate differences between trials.

(B) Differences between clones: Significant deviance would indicate significant differences between the dose response of the aphid clones tested.

Table 3.5 Probit analysis of the dose response between inner and outer zone for clones S, R1, R2 and R3 sprayed *in situ*.

Between zones (inner - outer)					
Clone	Single line model			Parallel lines model	
	d.f.	*Deviance*		*d.f.*	*Deviance*
S	7	12.26		-	-
R1	8	27.78	*	7	9.62
R2	7	22.53	*	6	2.54
R3	8	20.90	*	7	7.68

The deviance was estimated using the single linear model that would indicated differences between the zones. In the case of significant deviance the parallel lines model was also used. Combination of the models would indicate the type of dose response differences between the zones. Significant deviance is followed by (*) for $P<0.05$.

Probit analysis in the Genstat procedure library was used, to estimate the median lethal concentration for each aphid clone (see section of Statistical analysis in Chapter 2). Based on findings that the mortality recorded was affected by the position on the repli dish, the recorded dose response was analysed by looking separately at the inner and outer zones on the dish, as well as looking at the whole dish (Table 3.4-*A*). In this way, the analysis was done including ('all dish') or excluding ('inner' and 'outer' zone) the factors (related to position) affecting the mortality. No statistically significant differences were observed between trials for any of the 4 clones. The LC_{50} calculated for the whole dish was in-between the two estimates for the inner and outer zone. The concentration needed to kill 50% of the population in the inner zone (centre of dish) was always the lower, which is consistent with the findings of retrospective analysis (see section 3.3.2). Indeed, further analysis 'between zones' for individual clones indicated that the dose response in the inner zone was significantly different from the outer zone (Table 3.5). Clone S did not show significant deviance between zones but the value was high (for $d.f.=7$, $X^2=14.07$ at $P<0.05$). The data between zones from clone R1, R2 and R3 fitted the parallel lines model. This suggests that the dose

response of the inner and outer zone had a similar spread, but they were comparatively transposed. Nevertheless, it is important to observe that there was no significant deviance between the trials, when the inner and outer zones were tested separately (Table 3.4-*A*). This indicated that the factors affecting mortality (in relation to position on the repli dish) had a similar effect on the different trials.

No statistically significant differences were observed between the clones when the data were analysed by zones (Table 3.4-*B*). This finding allowed the comparison between the clones over all. No statistically significant deviance was observed over all. A common LC_{50} (2.7×10^5sp/ml) was calculated by the program for all aphid clones.

3.3.3.2 'Secondary pick up' experiment - 7 day exposure

This experiment was designed to compare the secondary spore pick up of the four aphid clones (S, R1, R2 and R3), over a long time period. The repli dish was sprayed with 10^6sp/ml, and the aphids were allowed to pick up spores through out the incubation period (7 days). The mortality after a 7-day incubation is illustrated in Figure 3.27. The differences between the clones were not significant.

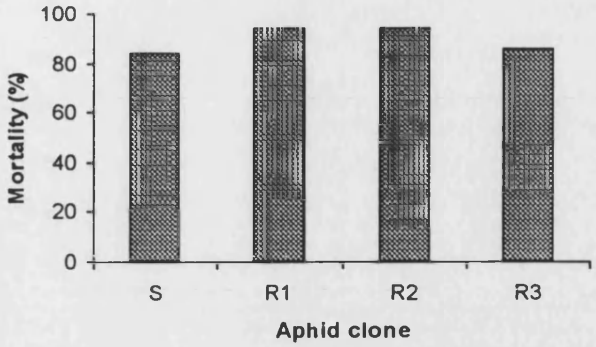


Figure 3.27 Aphid mortality for clones S, R1, R and R3 in 'secondary pick up' experiment - 7-day exposure.

Aphid mortality after 7 days of exposure on infected surface is displayed (n=50 per treatment, applied dose: 10^6sp/ml). No control mortality was observed. A X^2 test was applied to the raw data. No statistically significant differences were observed between the clones (df=3, P<0.05).

3.3.3.3 'Secondary pick up' experiment - 24 h exposure

This experiment was designed to compare secondary spore pick up of aphid clones R1 and R3 over a 24h interval. The amount of inoculum may vary, depending heavily on the activity level exhibited by each aphid clone. After the Petri dishes were sprayed with spore concentration ranging from 10^5 - 10^8sp/ml, the aphids were applied to the infected surface for 24h. Then, the insects were transferred to individual uninfected chambers, and were incubated for a further 6 days interval. The data are displayed in Figure 3.28. Both clones showed a significant correlation between mortality (%) and applied spore concentration (P<0.05). The highest mortality (80%) was recorded for concentration 10^8sp/ml.

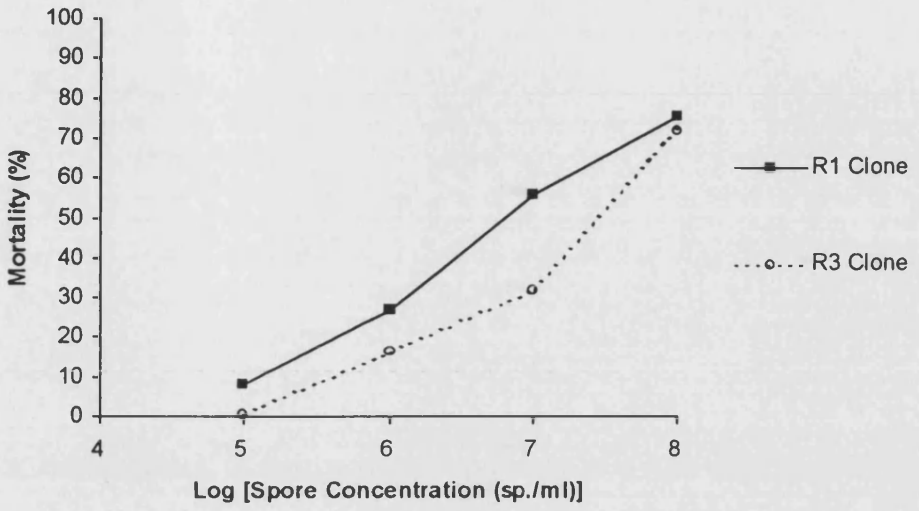


Figure 3.28 Comparing aphid mortality (%) of clones R1 and R3 in 'secondary pick up' experiment - 24-h exposure.

Aphids (n=50 per treatment) were applied for 24 h on leaves infected with a spore suspension ranging from 10^5 - 10^8sp/ml. The mortality (%) recorded after a further 6 day incubation is illustrated on the graph. No control mortality was observed. The points represent the mortality recorded overall. Statistical analysis of the dose response (probit analysis) showed no significant differences between the aphid clones (see Table 3.6-*B*) ($P<0.05$).

Probit analysis on the Genstat was used to estimate the LC_{50} for each aphid clone (Table 3.6-*A*). No statistically significant deviance was observed between trials for any of the clones when the data were analysed by zone or, when the plate was tested overall. This suggests that: a) factors affecting the mortality (in relation to position) had similar effects on the different trials for a clone and, b) dose response was similar over all, among the trials for each clone. The LD_{50} calculated for the inner zone was always the lowest one, which is in agreement with the result from the retrospective analysis (see section 3.3.2). Further analysis indicated that the dose response between zones was not significantly different (Table 3.7), suggesting that the factors affecting

mortality, in relation to position, did not have a significant effect in 'secondary pick up' experiments.

Table 3.6 Probit analysis of the dose response recorded for clones R1 and R3 for secondary spore pick up application method.

Within clones

Clone	Zone	Log(LC_{50})	s.e.	*d.f.*	Deviance	
R1	*Inner*	7.16	0.22	8	6.93	*A*
	Outer	7.85	0.20	8	4.62	
	All dish	7.59	0.15	8	0.84	
R3	*Inner*	7.98	0.18	10	11.14	
	Outer	8.14	0.16	10	8.51	
	All dish	8.08	0.12	10	11.45	

Between clones

Clones	Zone	Log(LC_{50})	s.e.	*d.f.*	Deviance	
R1 and R3	*Inner*	6.96	0.14	8	10.74	*B*
	Outer	7.35	0.13	8	6.06	
	All dish	7.16	0.09	8	13.90	

The LC_{50} was estimated separately for each zone and for the whole repli dish. The deviance was estimated using the single linear model that would indicated differences between the treatments. Significant deviance is followed by (*) for $P<0.05$ (*s.e.* for standard error).

(A) Differences within a clone: The variation between the trials (dose response experiments) for a clone was estimated. Significant deviance would indicate differences between trials.

(B) Differences between clones: Significant deviance would indicate significant differences between the dose response of the aphid clones tested.

Table 3.7 Probit analysis of the dose response between zones (inner - outer) for clones R1 and R3 for 'secondary pick up' application method.

Between zones (inner - outer)		
Clone	*d.f.*	Deviance
R1	8	6.11
R3	8	4.51

The deviance was estimated using the single linear model that would indicated differences between the zones. Significant deviance is followed by (*) for P<0.05.

No statistically significant differences were observed between the aphid clones (Table 3.6-*B*). Analysis of the data by zone showed a similar impact of the factors affecting the mortality (in relation to position on the repli dish). This finding allows the comparison between the clones over all. A common LC_{50} (2.2×10^7sp/ml) was calculated by the program for the two aphid clones.

3.3.3.4 'Direct impact' experiment

This experiment was designed to compare the susceptibility of the aphid clones to *V. lecanii*, when a known amount of inoculum was deposited. Aphids were sprayed with spore suspension ranging from 10^5 to 10^8sp/ml. The insects were then transferred to individual uninfected chambers. The mortality after 7-day incubation interval is displayed in Figure 3.29. Both clones showed a significant correlation between mortality and the applied spore concentration (P<0.05). The highest mortality (83%) was recorded for the concentration 10^8sp/ml. Low control mortality was observed (2%) for clone R1.

Probit analysis in the Genstat procedure library was used to estimate the LC_{50} for each aphid clone. Statistically significant deviance was observed between the trials for both

aphid clones (Table 3.8-*A*). When the data were analysed separately in zones, significant deviance was again observed. This suggests that the factors responsible for the observed variability were not related to the position on the repli dish.

No statistically significant differences were observed between the two clones when the data from all experiments were combined (Table 3.8-*B*). Dose response analysed by zones also did not indicate any significant deviance. This suggested that some factors affected mortality randomly, but the over all response of the clones was similar. A common LC_{50} (6.2×10^6sp/ml) was calculated by the program for the two aphid clones.

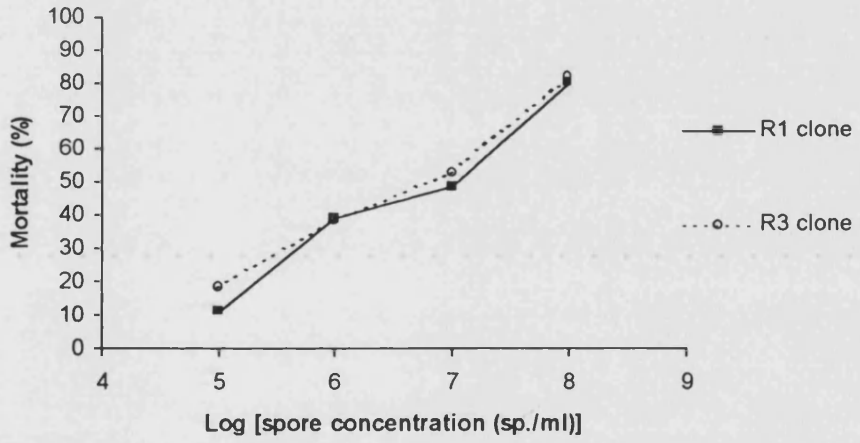


Figure 3.29 Aphid mortality for clones R1 and R3 in 'direct impact' experiment.

Aphids (4 replication, 25 per treatment) were sprayed directly with spore concentrations ranging from 10^5 to 10^8sp/ml, and were transferred to uninfected individual chambers within 20 min. The mortality (%) recorded after a 7 day incubation is illustrated on the graph. The points represent the overall mortality. Abbot's formula was used to correct for control mortality (see Chapter 2). Statistical analysis of the dose response (probit analysis) showed no significant differences between the aphid clones (see Table 3.8) ($P<0.05$).

Table 3.8 Probit analysis of the dose response recorded for clones R1and R3 for 'direct impact' application method.

Within clones — *A*

Clone	Zone	Log(LC_{50})	s.e.	d.f.	Deviance	
R1	*Inner*	7.38	0.17	18	37.77	*
	Outer	7.61	0.14	18	44.82	*
	All dish	7.52	0.11	18	55.07	*
R3	*Inner*	6.90	0.19	18	33.96	*
	Outer	7.41	0.15	18	23.77	
	All dish	7.21	0.12	18	49.74	*

Between clones — *B*

Clones	Zone	Log(LC_{50})	s.e.	d.f.	Deviance
R1 and R3	*Inner*	6.57	0.12	8	14.10
	Outer	6.93	0.10	8	6.80
	All dish	6.79	0.08	8	15.01

The LC_{50} was estimated separately for each zone and for the whole repli dish. The deviance was estimated using the single linear model that would indicated differences between the treatments. Significant deviance is followed by (*) for $P<0.05$ (*s.e.* for standard error).

(A) Differences within a clone: The variation between the trials (dose response experiments) for a clone was estimated. Significant deviance would indicate differences between trials.

(B) Differences between clones: Significant deviance would indicate significant differences between the dose response of the aphid clones tested.

Calculating median lethal dose for 'direct impact' experiment

The spores that are deposited on the aphid using the 'direct impact' application method for a given spore concentration ranging between 10^5 to 10^7, can be estimated from the fitted linear equations displayed in Figure 3.15. Using this formula, the median lethal concentration (LC_{50}) previously calculated using probit analysis, could now be

transformed to median lethal dose (LD_{50})[1]. However, the calculation has to be done in steps that are explained in the flowing text.

LC_{50} was previously calculated using probit analysis. The LD_{50} was now estimated using the data presented in Figure 3.15 and Figure 3.21. The linear regressions transform spore concentration (C in sp./ml) to deposition (N in sp./mm^2) and the dose (D in sp./aphid) could be estimated by multiplying deposition with the aphid surface (see section 3.3.1.1 and Figure 3.15). Using the fitted linear regression we can only estimate the number of spores on the dorsal side of the aphid (D_d). However, experiments on allocation of spore deposition when using 'direct impact' application method (see section 3.3.1.8 and Figure 3.21) showed that 88% of the total number of spores are located on the dorsal side of the aphid. This suggested that the number of spores on the dorsal side of the aphid using 'direct impact' application method was a known proportion of the total number of spores on the insect.

Calculating deposition (N):

For concentration (C) = 6.2×10^6sp/ml (LC_{50}) or Log(C) = 6.79 the result from the linear equation is:

Log (N_{dorsal}) = 1.91 or:

N_d = 81.5 (sp./mm^2)

Calculating dose (D):

$D_d = N_d \times$ (aphid dorsal surface)

(aphid dorsal surface) = (total aphid surface)/2 = 1.17 mm^2 (see section 3.3.1.1)

Dose on the dorsal side (D_d) = 95.4 (sp./aphid)

Scaling up to the applied dose (total number of spores deposited):

$D_T = D_d/0.88$ = 108.4 (sp./aphid)

For application method 'direct impact' the LD_{50} is equal to 108.4(sp./aphid).

[1] The dose (spores/aphid) required to kill 50% of the treated population.

3.3.3.5 Comparison between the application methods

The efficiency of the three application methods is compared using probit analysis (see statistical analysis Chapter 2). The data from the *in situ* method, 'secondary pick up', and 'direct impact' for clone R1, are plotted together in Figure 3.30. The probit analysis program on Genstat showed that the *in situ* method was significantly more efficient, than the other two (Table 3.9). The dose response for 'secondary pick up' and 'direct impact' did not differ significantly.

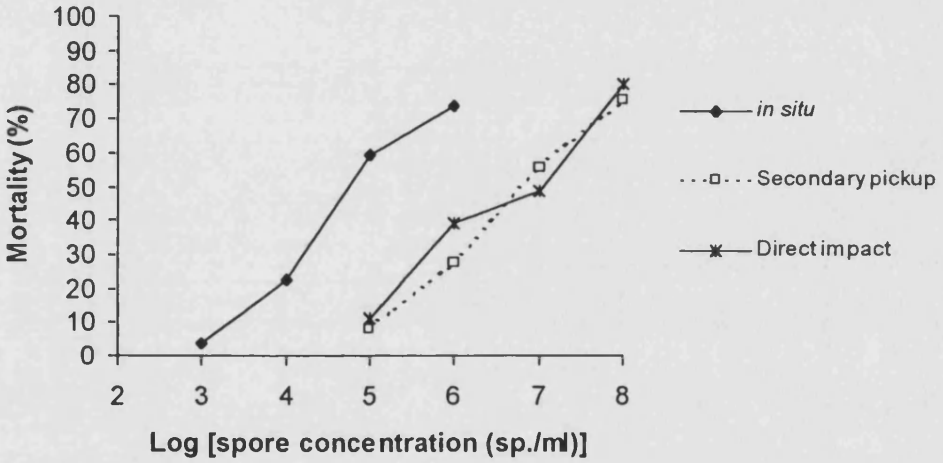


Figure 3.30 Comparison between the three spore application methods for aphid clone R1.

The mortality recorded after 7 days of incubation using concentrations ranging from 10^3 to 10^8sp/ml is displayed on this graph for the application methods *in situ*, 'secondary pick up' and 'direct impact'. The points represent the over all mortality recorded in each experiment.

Table 3.9 LC_{50} estimated with probit analysis for three spore application methods (aphid clone R1).

Method	*LC_{50}*	*s.e.*	
In situ	9.23×10^4	3.04×10^4	*a*
Secondary pick-up	876.38×10^4	208.65×10^4	*b*
Direct impact	825.22×10^4	263.86×10^4	*b*

LC_{50} in (sp./ml). Application methods with the same letter are not significantly different ($P<0.05$).

3.3.4 Results from horizontal spore transmission experiment

The experiment was designed to study the case of spore transmission from a mycosed insect to other neighbouring insects. The effect of the exposure interval with the mycosed insects on the mortality was investigated. The duration of exposure varied between 1-3 days. Furthermore the disease development of the mycosed aphid, (different inoculum levels) in relation to the mortality of the untreated aphids, was studied. To achieve the different levels of fungal inoculum, untreated aphids were placed together with infected aphids, which were previously incubated for 1 day (germinating conidia), 3 days (beginning of sporulation) or 7 days (sporulating cadaver). The mortality (%) after a total incubation of 7 days is displayed in Table 3.10. The data showed a strong correlation, both with exposure interval, and with level of infection (incubation of infecting aphid).

Table 3.10 Aphid mortality (%) by horizontal spore transmission for aphid clone R1.

		Exposure interval (days)			*r*
		1	*2*	*3*	
Incubation of infecting aphid (days)	*7*	35.6	61.7	72.9	*0.97*
	3	33.3	35.0	47.8	*0.91*
	1	11.0	5.9	22.0	*0.67*
	r	*0.81*	*0.98*	*0.98*	

Aphids (n= 50, 7 day, n= 25 for 1 and 3 days) were incubated together with a mycosed aphid for 1,2 or 3 days. The infection of the mycosed aphid was allowed to develop for 1, 3 or 7 days (different levels of inoculum). The mortality (%) after a total of 7 days incubation is displayed in the table. A strong correlation (r) can be observed between mortality - exposure interval and mortality - infection development.

3.4 Discussion

3.4.1 Preliminary experiments

The estimate of the aphid surface using image analysis (1.84mm^2) is close to the results from the 3D model (2.35mm^2). However, the image analysis is considering the aphid as 2 dimensional, (flat surface). The 3D model is overcoming that error, giving a better estimate of the surface.

A significant decrease in the number of spores deposited, in relation to the distance from the centre of spray disk was observed, when using the Potter tower for spore application. However, the spore deposition was not different for any position with the same radial distance from the centre of spray disk. Although Potter (1952) suggested that this system produced an homogenous spray deposition, this was not true for the equipment used in this project, despite the fact that it was serviced prior to the project.

Retrospective analysis of data from 50 experimental trials revealed differences in the recorded mortality, in relation to the position of the subject (aphid), in the repli dish (edge or centre). These differences were associated with effects of ventilation on the RH of the cells. These differences were intensified when *in situ* ('spray dependent') experiments were carried out. This observation was associated with an additional differentiation factor; the heterogeneous spore deposition during *in situ* application discussed previously.

Once these variability factors in the bioassay trials were highlighted, the data analysis for the bioassays was adjusted, to test the level of significance, and the consistency of

the resulting variability. If these factors (affecting mortality in relation to position within the repli dish) were consistent and affected all experiments, comparisons between treatments would still be valid. Concerning the spore deposition difference between the centre and the side of the bioassay dish in 'spray-dependent' experiments, the deposition increase was 2-fold (maximum). In dose response experiments, the typical step between treatments is a 10-fold increase in spore concentration. Such an increase in spore concentration (and deposition) outweighs the variability of spore deposition.

There was a significant correlation between the spores deposited on glass cover slips or the dorsal aphid body side and the spore concentration of the spray suspension. Linear regression fitted the data well both for glass and aphid (dorsal) surfaces, allowing calculation of the spores deposited on the respective surfaces.

The spore deposition on the ventral aphid side also showed a correlation with the applied spore concentration. However, in this case the data showed higher variation. Accidental events, like contact with a spray droplet on the leaf surface, can increase the spore pick up and the variation. More important though, in the author's opinion, are the differences of the activity levels of the insects. Hall and Papierok (1982) pointed out the dependence of aphid control with *V. lecanii* on contagion while the insect is mobile. However, their observations were restricted to the resulting mortality (%), rather than spore deposition itself.

Use of Triton X-100 did not affect the efficacy of *V. lecanii* application in the current bioassay system. Hall (1982) reported that infectivity of *V. lecanii* was impaired when

spores were suspended in Triton X-100 compared to suspension without the wetting agent. These findings, in combination with the inability to evaluate spore deposition in the presence of the wetting agent, supported the use of plain dH_2O, in future spore suspension applications.

50% spore germination on BPA at 24°C was estimated at 7.9h. Similar experiments by Hall (1977), at 24°C and on SDA (Sabouraud dextrose agar; 10g/l mycological peptone, 40g/l glucose 15g/l agar) showed a 6h period required for 50% germination.

Good spore germination, reaching 95% was observed on aphids after 24h incubation, and under optimum humidity conditions. The dependence of *V. lecanii* spore germination on free water availability has been demonstrated on media (Chandler *et al.*, 1994), on live insect specimens (Chandler *et al.*, 1993b; Drummond *et al.*, 1987; Hall and Papierok, 1982), and in bioassays (Hall, 1981; Milner and Lutton, 1986).

Surface growth occurred prior to and after cuticular penetration. The extent of surface growth, prior to death, varied considerably in early experiments. Extensive hyphal growth of *V. lecanii* on cuticular surface has been observed many times (Drummond *et al.*, 1987; Schreiter *et al.*, 1994; Sitch and Jackson, 1997). Brey *et al.* (1986) suggested that the high nutrient status of the aphid cuticle, from honeydew secretion, encouraged the saprophytic surface growth of the fungus. They showed that, honeydew stimulated the conidia germination of the aphid pathogen *Conidiobolus obscurus*. Schreiter *et al.* (1995) suggested that the cuticle degrading ability of *V. lecanii* on western flower thrips is used, to release nutrients, and encourage mycelia

growth rather than invasion and penetration of the aphid cuticle. Death occurred from damage of the cuticle and stress of the host.

In the present work there was evidence of restricted surface growth on honeydew free aphids. Short germ tubes and penetration pegs were observed under the Cryo. SEM system. This suggests that honeydew is probably the factor stimulating surface growth, rather than the release of nutrients from the enzyme activity of the fungus. However, aphids are not honeydew free in the field.

Lower and variable germination was observed on the ventral side, compared to the dorsal side of the aphid body. There are two possible, complementary explanations for this:

a) The spores on the dorsal aphid side were attached to the cuticle during spray application; synchronous germination followed (see results in section 3.3.1.8). The spores on the ventral side were acquired by 'secondary pick up' through out the incubation period. The germination would appear protracted and less efficient.

b) The number of spores found on the ventral side is lower that the one found on the dorsal. A smaller size of spore sample for each observation can produce high variation.

The number of spores counted on aphid bodies from the 'secondary pick up' experiment showed great variability. However, the location of patches of spores on the aphid body, and in particular on the legs, is consistent with accidental pick up following aphid movement.

The mortality recorded in secondary pick experiments was positively correlated with the exposure time. Milner and Lutton (1986) also for aphid *M. persicae*, found the same correlation between exposure time and mortality in secondary spore pick up bioassays using whole plants (peppers) treated with Vertalec. Secondary spore pick up lead to an almost continuous acquisition of fungal inoculum. These findings help to explain the high variance of spore germination, on the aphid's ventral side, discussed previously (results in section 3.3.1.6). .

Nevertheless, *V. lecanii* spores applied to pepper leaves rapidly lost their viability. There was a significant negative correlation with time. Within 3 days more than half of the spores were unable to form colonies (compared to number of *c.f.u.* recorded right after application). A comparative reduction in killing power was observed with time in 'secondary pick up' experiments. There was a negative correlation between the time that the aphids were introduced to the infected leaf surface and the mortality %. These findings suggested a restricted exposure interval in 'secondary pick up' bioassays, (the first 24h) to avoid additional variability parameters (like loss of spore viability with the time) in the experimental design.

Secondary pick up also depends on microclimatic conditions, especially humidity. Hall (1979) discussed the effect of leaf surface moisture on spore pickup by aphid *Macrosiphoniella sanboni*. He suggested that the pick up from fully dried leaves was reduced, compared to leaf surfaces that were not dry. In all secondary pick up experiments the conditions were preserved under high humidity. However, the leaf surface itself was left to dry for 10 minutes to avoid the presence of droplets.

Preliminary secondary pick up experiments indicated that droplets increase the result variability.

3.4.2 Comparative studies on the aphid clones

It was demonstrated that the mortality recorded in bioassay trials was affected by factors related to the position in the repli-dish. Data analysis by zones (inner and outer) was used to study the consistency of these factors, for *in situ* and secondary pick experiments. Indeed, the effects of these factors were consistent between trials for single clone experiments, and between all clone experiments. This consistency suggested that the recorded dose responses for the aphid clones studied were subjected to the same variability factors, and therefore these results were comparable.

In situ experiments clearly illustrated no significant differences in the recorded dose responses among all 4 aphid clones. The spore pick up in these experiments, depends, both on direct contact with sprayed suspension, and on secondary pick up. Therefore, absence of differences in the dose response indicates possible absence susceptibility differences between the clones. However, this was to be investigated further by experiments on secondary pick up and direct impact.

There was evidence that the differences between the zones in 'spray dependant' experiments were greater, compared to 'spray independent' experiments (section 3.2.3). Indeed, analysis of the dose response between zones in *'in situ'* ('spray dependent') experiments indicated significant differences for 3 out of 4 clones (Table 3.5). No dose response differences between zones were observed in 'secondary pick up' ('spray independent') experiments (Table 3.7). The combination of higher

deposition and favourable humidity conditions in the inner zone affected the dose response in 'spray dependant' (*in situ*) experiments. In 'spray independent' ('secondary pick up') experiments, humidity was the only differentiating factor affecting the mortality differences between the zones. The effects were consistent, which was in agreement with the retrospective analysis, but not significant.

The effect of the zones in repli dish bioassays has not been reported before. However, in experiments with a similar set up only the 9 central cells were used for assessment of mortality (Chandler *et al.*, 1993b) or, the whole set-up was placed in a container over water (Jackson *et al.*, 1985) or, over salt solutions, when the effects of reduced humidity were studied (Drummond *et al.*, 1987). Using these methods they avoided the zone effect. Chandler *et. al* (1993a) though, did not use any of the above methods in the bioassay set up but the zone effect was not observed. However, in that particular set up, the implemented method was 'spray independent', and any effects would probably be not significant.

The 'direct impact' experiment was designed to aim exclusively on the susceptibility differences between the two extremes of the resistant aphid clones (R1 and R3). Although variability was observed for the 'direct impact' experiment between trials, this was not related to the side effect variability factors. Data analysis in zones also highlighted a similar variability, as the one observed for the overall repli-dish analysis. However, looking at the resulting dose response for the two clones over all, no significant deviance was observed. The clones responded to the applied inoculum in the same manner. The over all susceptibility of the clones was not significantly different.

Using the linear regressions to estimate the relation of applied dose and spore deposition on aphids (see section 3.3.1.3), it was possible to transform the LC_{50} to LD_{50}, for the application method 'direct impact'. The LD_{50} was estimated at 108.4 spores/aphid. These results suggest that only few conidia per adult are required to cause infection in an aphid population under optimum conditions. Moorhouse (1990) found a similar low LD_{50} (138 sp./adult) in bioassays involving *M. anisopliae* against the vine weevil. However, studies have shown greater variation in LD_{50} between fungal species (Doberski, 1981).

Unfortunately, relation of dose and spore deposition in 'secondary pick up' experiments was not established. Therefore, it was not possible to estimate the LD_{50} for application methods that involve secondary pick up of spores (*in situ* and 'secondary pick'). Nevertheless, using the linear equations in section 3.3.1.3 it was possible to estimate a spore deposition on the sprayed surface, in relation to the LC_{50} for the 'secondary pickup' method; a spore deposition of 33.3 sp/mm^2 was shown to kill 50% of the population. Although the conditions in the field are much different and variable compared to laboratory (e.g. temperature and humidity fluctuations, low homogeneity of the spray application), these estimations between spore deposition and aphid mortality can be used as a common ground, to associate field and lab results in future trials.

Here, I would like to point out, an interesting observation concerning the 'direct impact' and 'secondary pick up' experiment. In 'direct impact' experiments a relation between spore-attachment and spore concentration was established (3.3.1.8), something that was not possible for application method 'secondary pick up' (see

3.3.1.9). In 'secondary pick up' experiments spores could be found on the aphids only when the spore suspension with the highest concentration (10^8sp./ml) was applied, and still only highly variable, low numbers of spores were counted. Nevertheless, a similar dose response relationship was observed (Figure 3.30) and similar LC_{50} values were estimated for both application methods (8.7 and 8.2x 10^6sp/ml). This indicates that a very small number of spores are needed for a successful disease development in 'secondary pick up' experiments. This highlights the importance of secondary pick up as an infection pathway. The absence of spores on the aphid body when low concentrations where applied also indicates that infection occurs from a pathway other that the body cuticle. That pathway is also more efficient as a low number of spores are necessary. The part always in contact with the inoculated surface are the legs. The joints, especially that of the tarsus could possibly provide an easy access in the aphid body to the pathogen.

Foster *et al.* (1996) suggested that some unknown selection pressure countered evolution of insecticide resistance in the field, after measuring a reduced proportion of resistant aphids in field trials during the winter period. This was associated with poor survival of the resistant aphids in low temperatures, but they also showed that overproduction of esterase was not the reason for these observations (Devonshire *et al.*, 1998; Foster and Harris, 1997). Up to now, overproduction of the enzyme esterase in resistant aphid clones has not been associated with any differences between the resistant and susceptible clones other than tolerance to insecticides. The present work, in agreement with the above authors, provided evidence of absence of relation between overproduction of esterases and susceptibility to fungal infections.

Apart from the dependence of secondary pick up on factors discussed in the 'preliminary experiments' section (applied dose, exposure interval and environmental conditions) it is also believed to be associated with activity levels. Susceptibility differences in field trials have been associated with behavioural differences between aphids species and the resulting restricted secondary spore pickup, with the inoculated vegetation (Hall, 1981; Hall and Papierok, 1982; Milner and Lutton, 1986). However, this assumption is not based on experimental evidence relating insect activity levels with spore pick up or mortality.

'Secondary pick up' experiments (both *24h and 7 day exposure*) were applied to the aphid clones and were designed to investigate any differences in the spore pick up and in the clone susceptibility. Given that in the previous experiment the susceptibility of the two clones to fungal infection did not differ, any dose response differences ought to be accounted for by other factors (behavioural differences). However, probit analysis showed no significant differences between the two aphid clones, suggesting possible absence of behavioural differences between R1 and R3 clones, under optimum laboratory conditions.

Comparison of the application methods clearly illustrates the importance of secondary pick up in inoculum acquisition. 24 h of spore pick up, under optimum environmental conditions is as effective as directly hitting the aphid with spore suspension. The significant mortality difference between the *in situ* method and 'direct impact' method is accounted for by such additional spore pick up, during the experiment, from the infected leaf surface. Preliminary experiments provided additional evidence to associate aphid mobility and exposure interval to increased secondary spore pick up.

Studies on aphid behaviour (see Chapter 4) and methods for manipulation (see Chapter 5) are possible ways to improve aphid control with *V. lecanii*, by exploiting the suspected relation of spore pick up with the activity levels of the insect.

3.4.3 Horizontal spore transmission

Dispersal of infectious propagule by fungal pathogens is mainly passive and depends on environmental factors. Few insect pathogenic species forcibly discharge their conidia or produce actively dispersing spores (Hajek and St Leger, 1994). Aerial dispersal for *V. lecanii* is not considered possible (Hall, 1981) and infection depends on accidental contagion. Hall (1984) investigated the epizootic potential of *V. lecanii* isolates (here defined as 'horizontal transmission'[1]). It was demonstrated that strong epizootic potential was achieved by fast germinating isolates.

Hall (1984) suggested that a pathogen could be successful against a rapidly reproducing pest, like aphids, if it possesses the ability to spread efficiently through the host population. In the present work, the ability of *V. lecanii* (Vertalec) spores to be transmitted horizontally, in an aphid population in relation to the time and the development stage of the disease was investigated.

Mortality % caused by horizontal spore transmission showed good correlation both with exposure time, with the infecting insect and with the state of the infecting insect, that is directly related to the level of infective inoculum. The presence of infecting

[1] The case of infection spreading to an aphid population from a dead sporulating aphid cadaver, (infection unit)

inoculum only 1 or 2 days after application, can only be attributed to the spray application, or by conidia production from saprophytic (surface) growth. *V. lecanii* has been reported to show extensive surface growth (Schreiter *et al.*, 1994). Conidia formation after just 2 days of incubations has been observed by Graystone (Ph.D. thesis in prep.) and the author for *V. lecanii* (isolate Vertalec).

Possible ways that uninfected aphids could acquire spores from the sporulating aphids are the following:

a) the spores were detached from the sporulating aphid while transferred or during incubation; detached *V. lecanii* spores on leaves were shown to survive for a few days (see section 3.3.1.11),
b) uninfected aphids accidentally came in contact with the infecting aphids, which were observed a in a few cases during the experiment preparation.

CHAPTER FOUR

Comparative studies on aphid behaviour

4.1 Introduction

Insects perceive their external environment through sensory organs (sensilla) that protrude through or lie beneath an insensitive protective barrier, the cuticle. The detected stimuli can be categorised as mechanical, thermal, chemical or visual. Sensilla translate the stimuli into neuronal reflexes and information is integrated by a central nervous system that instigates and controls the insect's behaviour (Gullan and Cranston, 1994).

According to Matthews and Matthews (1978) the insect's activities can be divided into maintenance and communicatory activities. Locomotion, grooming and feeding can be considered as maintenance activities that serve for the wellbeing of the individual. Actions that aim to convey information and influence the activities of others are characterised as communication activities and include chemical, mechanical and visual communication codes. Defensive, reproduction and social behaviours are all mediated by the insect's communication activities.

Most insect behaviours are considerate to be innate[1]. However, it has been shown that environmental conditions, physiological state of the insect, past experience and learning, can modify otherwise stereotypic behaviours (Du *et al.*, 1997; Grasswitz, 1998; Gullan and Cranston, 1994; Vet and Groenewold, 1990). The great variety of behaviours established by the insects (including the behavioural plasticity), in their way all have a survival value, and ultimately contribute to the optimisation of the insect's reproductive success (Matthews and Matthews, 1978).

As befits their status as major crop pests aphids have been the focus of much research. Aphid life cycles were described in detail in the relevant section of Chapter 1. In this section, aspects of the behaviour of viviparous apterae of *Myzus persicae* are reviewed.

Feeding behaviour

Aphids are plant sucking parasites that penetrate plant tissue with their stylets and feed on the phloem sap. When aphids visit a new host they need time to recognise the suitability of the host mainly by the structure and chemistry of the surface and the internal tissues (Dixon, 1998). Aphids show a characteristic behaviour consisting of walking and probing[2]. They scan the surface with their proboscis and positive stimuli from the host increase the duration and frequency of probes and walking time becomes shorter (Klingauf, 1987b). Only after repeated exploration of the feeding source will individuals attempt to settle and fed on the host (Klingauf, 1987a). The feeding site is selected according to feeding preferences and development stage of the

[1] Genetically programmed behaviour demonstrated once exposed to the appropriate stimulus
[2] Brief and short penetration (average duration 1min) insufficient for feeding, used for host recognition

aphid and is also affected by the physiological state of the host (water content) (Klingauf, 1987a). As with most aphids *M. persicae* penetration will typically end in the phloem and very rarely in the xylem (Esau *et al.*, 1961). A preference is shown for young (growing) or senescent leaves, based on the nutritional value of the sap (Kenedy *et al.*, 1950). Adaptation to a new host can take several hours (4 – 8h) (Schnorbach, 1983).

Once aphids find a suitable feeding site they penetrate the plant with their needle-like mandibular and maxillary stylets. Ability to penetrate and reach the food source is of vital importance and early instars are equipped with disproportionately long stylets (Schnorbach, 1983). As the penetration of the plant tissue begins, a salivary sheath is continuously secreted, that gives rigidity to the flexible stylet and control over the direction of the probe (Miles, 1987; Pollard, 1973). It takes about one minute to penetrate the plant epidermis, and 15 minutes to several hours to reach the phloem (Klingauf, 1987a). Using an electrical penetration graph (EPG) (as described by McLean and Kinsey, 1964) the intercellular pathway followed by the stylet during penetration has been determined (Tjallingii and Hogen Esch, 1993). Watery saliva is pumped into the wounded cell (phloem element) possibly to slow down the cell's defences. Saliva components have a buffering effect on aggressive plant substances, and may also divert nutrients away from growing parts of the plant. This 'sink effect' provides the aphids with a continuous flow of nutrients (Way, 1967). The sap pressure (15-30atm.) is sufficient for food uptake. However aphids can control the flow (Dixon, 1998; Klingauf, 1987a).

As the sap enters the insects gut, its initial high osmotic pressure is reduced by converting the mono and disaccharides into oligosaccharides, actively reducing the number of molecules in the solution. Aphids equipped with a filter chamber are also able to dilute the incoming sap using the 'processed' low osmotic pressure liquid of the hindgut (Dixon, 1998).

Aphids are limited by dietary nitrogen. They assimilate about 60% of the nitrogen content of their food, which is very efficient, considering the large amounts of sap that aphids have to process (around their own weight in sap per day). The quantity of sap accumulated by aphids is related to the nitrogen content (Dixon, 1998). Growth and reproduction rates were related to increasing concentration of nitrogen in artificial diets (Dadd and Mittler, 1965).

Estimates of feeding rate and consumption of sap by aphids can be based either on measuring the ingestion of sap through cut stylets or by determining the excretion of honeydew. The most accurate method combines both procedures (Klingauf, 1987a)

Excretion

Honeydew is defined as the production of excretory droplets through the anus of aphids and other plant-sucking insects. It is a mixture of a large variety of compounds. High sugar content is typical (fructose, glucose, sucrose, and other oligosaccharides) at concentrations ranging from 1-10%. About 20 amino acids have been detected, most of them found also in the plant sap. The composition of honeydew depends on the quality of food and on seasonal changes. Honeydew attracts about 246 insect species and is vital for the survival of certain ant species (Klingauf, 1987a).

The aphid colony is protected from its own sticky honeydew excretion because droplets are sprayed a few centimetres away from the colony. Before spraying the body is held erect. The mechanism of droplet detachment is not completely understood. Honeydew excretion rate depends on the instar, on the host plant and its physiological status, and on environmental factors like temperature, humidity and time of the day (Klingauf, 1987a). Honeydew production can be used as an indicator of various events. Klingauf (1981) showed a relation between excretion and thunderstorms or changes in the atmospheric pressure. Excretion is mainly interrupted by moulting or changes of feeding site.

Locomotion

Aphids seem to be more adapted to a sessile than a mobile existence. Apterous aphids show only short periods of walking, and otherwise remain feeding almost continuously. Even winged morphs, with resources specially allocated to dispersal, sometimes perform only a few short flights before settling. When aphids walk, they keep three legs on the surface at all times like most insects. This gives them good stability and the ability to respond to danger at any time during their walking.

Dispersal

Aphid species monitor their environment from various cues and can determine when to disperse in search of more advantageous conditions. *M. persicae* mainly responds to food quality and tactical stimulation (crowding). Day length, temperature and non-nutritional plant factors affect the intensity of the response (Matsuka and Mittler, 1978). Apterae can only move to other locations on the host or to nearby plants. However a proportion of the progeny of dispersed apterae will develop wings (alatae);

morphs adapted for dispersal. The proportion of alatae morphs produced relates to the intensity of the stimulus (Dixon, 1998).

Defence

Apterous adult aphids under attack from a predator or a parasite can run away or drop off their feeding site. They have also been observed to kick and exude waxy secretion from the siphunculi over the attacker (Dixon, 1998). Aphids in danger can produce an alarm pheromone (E-β-farnesene) that alerts others individuals in the colony of a potential enemy (Pickett *et al.*, 1992). In response to the pheromone, aphids stop feeding, run away from the source or drop off the leaves.

Parthenogenetic reproduction

Aphids can achieve high reproductive rates by reproducing parthenogenetically. In part their pesticidal status can be attributed to their rate of increase. (Dixon, 1987). Aphids reach the highest reproductive rate early in their adult lives (Sylvester, 1954). However, each generation seems to have it's own reproductive strategy (size of offspring and timing of the peak in reproductive activity). This can be affected by extrinsic factors (food quality and temperature) and intrinsic factors (adults size and birth weight). Food quality has a direct positive effect on reproductive rate. Temperature has a similar effect within a limited range (up to 26°C for *M. persicae*) (Balrow, 1962; Dixon, 1987). Also the numbers of ovarioles and the morph of the adult (alatae or apterae) have an effect on the reproductive rate. Winged morphs produce less and smaller progeny. Aphids with large gonads (many ovarioles) can achieve higher reproductive rates, however, these individuals need longer

developmental times and show lower survival potential under poor habitats (Dixon, 1987; Dixon, 1998).

4.1.1 Genetics and behaviour

Nervous and muscular systems function in a precise way. Presence or absence of enzymes as a result of variability in the genome can have an impact on the neuromusculature systems and subsequently on the insect's behaviour. Benzer (1973) associated a mutated gene in fruit flies *Drosophila* sp., with behavioural differentiation. Crossing and selection experiments also established genetic determination of behavioural components. However, behavioural differences might not primarily be the result of the genetic variability (Matthews and Matthews, 1978).

Aphids show very little genetic polymorphism compared with that recorded for other insects (May and Holbrook, 1978; Suomalainen *et al.*, 1980). However, genetic variability does occur within and between species (May and Holbrook, 1978; Tomiuk and Wöhrmann, 1981). Variability in colour, host plant range, morphology, life cycle, and insecticide resistance have been associated with genetic variability (Dixon, 1985). Genetic change in asexual populations of *M. persicae* has resulted in failure to produce sexual forms in response to reduced day-length (Blackman, 1976).

Insecticide resistant *M. persicae* are tolerant to certain insecticides. The number of copies of the E4 gene encoding the detoxifying esterase correlates with enzyme activity in insect haemolymph and the extent of insecticide resistance (Devonshire and Field, 1995; Devonshire and Sawicki, 1979). In the absence of insecticides, resistant aphid clones showed decreased response to alarm pheromone (Dawson *et al.*, 1983).

Insecticide resistance has been associated also with poor survival under cold conditions, as these aphids were reluctant to move from deteriorating leaves (Foster *et al.*, 1996). Foster et al (1997) showed that the impaired perception of stimulus promoting movement, was not directly related to the overproduction of esterase. Recently however, Devonshire *et al.* (1998) concluded that the point mutation at the sodium channel (kdr) gene, responsible for knockdown resistance to pyrethroids, resulted in altered behaviour and perception of external stimuli. Amplification of the E4 gene is strongly linked to the kdr mechanism and other mutations (Field *et al.*, 1997) that could influence perception and locomotory behaviour (Foster *et al.*, 1997). Foster *et al.* (1997) also suggest that effects on the behaviour of resistant aphids can be a pleiotropic effect of DNA amplification (Field *et al.*, 1996) or transposition (Blackman *et al.*, 1995).

This chapter describes the behaviour of insecticide susceptible and resistant clones of *M. persicae* under optimal growth conditions.

4.1.2 Fungal infection and behaviour

Insect hosts respond to fungal infection by physiological and behavioural alterations (Hajek and St Leger, 1994; Tanada and Kaya, 1993). Horton and Moore (1993) reviewed the effects of parasites on the behaviour of their hosts. They included micro-organisms (fungi, viruses) and macro-parasitic organisms (parasitoids, nematodes) in their study. The behavioural alterations were categorised as (*a*) changes in microhabitat preferences, (*b*) changes in activity levels (*c*) effects on reproductive activity and (*d*) modifications of feeding behaviour (reviewed by Molyneux and Jeffries, 1986).

In several cases altered host behaviour is of potential benefit to the parasite and in other cases it benefits the host (Horton and Moore, 1993). Host behaviours allowing increased parasite dissemination (movement to elevated positions) and survival (movement to concealed locations) were considered to be controlled by the parasite. The same was interpreted for behavioural effects with potential benefit for the host (Krasnoff *et al.*, 1995). Movement to exposed locations may result in increased body temperature from solar irradiation with resultant 'fever' (Inglis *et al.*, 1996; Watson *et al.*, 1993). By using thermoregulation (behavioural fever), insects may combat the development of the pathogen and occasionally they recover. Behavioural effects that increase the host's inclusive fitness (survival of the kin) have been observed. In this case the pathogen-infected insect develops what is interpreted as host suicidal behaviour i.e. it moves to a new isolated habitats, reducing the chances of spread of the disease to the colony (Horton and Moore, 1993).

Some behavioural effects, like 'seeking of elevated locations', are induced by a wide range of parasites (parasitoids, fungi, viruses) suggesting that it is more like a general insect response to parasitism. Any benefits to the parasite might well be fortuitous (Horton and Moore, 1993).

Minchella (1985) suggested that behavioural effects from parasitism or infection may arise from natural selection favouring either the host or the parasite. Behavioural effects can also be a consequence of stress or pathological symptoms irrespectively of any benefit for either party.

There are a few reports on the effects of fungal infection on aphids. They all suggest that aphids tend to seek elevated locations once they are infected (Harper, 1958; Rockwood, 1950; Samson *et al.*, 1988). In this chapter the effects of fungal infection on aphid behaviour was studied on detached leaves in a controlled environment. Also *V. lecanii* isolates with different attack strategies (Bye, 1999, Graystone *unpubl.*) were used to investigate any differences in behaviour of mycosed insects.

4.1.3 Video techniques in behavioural studies on insects

Video techniques are particularly useful when observing organisms within a moderate sized arena and especially when the movement is restricted to two-dimensions (Varley *et al.*, 1994). Video filming allows repeated and detailed observations of insects activities, especially when events and interactions are happening simultaneously and it would be impossible to record them when working in 'real time'. It also allows observation of events happening very slowly or very rarely. Nevertheless a filming set requires careful design. Lighting must be adequate to allow good image quality, though light may itself influence behaviour. Increased temperature may result, in an enclosed arena, from heat given out by a light source. The size of the arena can be restricted by technical limitations but should be proportional to the subject's size, activity levels and the number of subjects. The shape of the arena is also important, as insects' tend to follow edges, which may influence the shape of the path. This must be kept in mind during data analysis. If the arena is not sealed, keeping the subjects in that area is an issue (Varley *et al.*, 1994).

Video filming can be successfully combined with computer analysis. Efforts to develop automated animal tracking date back a few years (Davenport *et al.*, 1961).

Successful electronic tracking of insects has been reported by Hoy, Globus and Norman (1983). Since then more advance computer analysis or computer tracking systems have been developed from animals, insects and micro-organisms (Chon *et al.*, 1997; Hader, 1994; Hoy *et al.*, 1996; Hoy *et al.*, 1997; Krens *et al.*, 1998; Vatine *et al.*, 1998).

Behavioural studies on aphids have been aided by video techniques in a few cases. These include studies on repellency (Nauen, 1995; Powell *et al.*, 1995a; Powell *et al.*, 1995b; Powell *et al.*, 1996; Powell *et al.*, 1998) and three-dimensional flight behaviour of alatae (Hardie and Young, 1997). In this chapter aphid behaviour was studied with time lapse video techniques supported by computerised analysis of behavioural data.

4.2 Materials and methods

4.2.1 Comparative experiments between aphid clones

4.2.1.1 Behaviour experiments

Aphid behaviour was studied using the video techniques described in Chapter 2. The methods developed for the behavioural analysis, visual observation of aphid behaviour and computerised tracking of aphids and their movement, were both employed in these studies.

For the needs of the visual observation experiments *'mobility'* was defined as that an rate of movement per day. Honeydew excretions have been used to estimate the feeding rate for aphids (Klingauf, 1987a). In these experiments honeydew drop excretion events are used as an indication of feeding activity of control and infected *M. persicae* adults.

4.2.1.2 Reproductive rate

Pepper leaf disks were embedded on water agar (1% w/v) in a 5cm Petri dish. One adult aphid was placed in each dish and was covered with the dish lid. The dish was incubated at temperature 24°C and photoperiod 16h L:8h D. The day that the first offspring was born was considered as Day 1. Offspring feeding relatively distantly from the adult were carefully removed with a paintbrush to avoid overcrowding. The number of offspring was recorded daily for five days. 7 aphids were tested for each aphid clone (S, R1, R2 and R3).

4.2.2 Effects of fungal infection on aphid behaviour

4.2.2.1 Behaviour experiments

These experiments were carried out as described in the relevant section in Chapter 2. Untreated insects (control) were sprayed *in situ* with 1.5 ml sterile dH_2O and treated insects (infected) were sprayed with 1.5 ml of 10^6sp/ml *V. lecanii* spores suspension. (isolates Vertalec and KV42).

Visual observation

Aphid clone R3 was used in these experiments. Low RH (room conditions) and high RH (saturated conditions) were tested. A filming arena (Petri dish) which was not sealed with parafilm® allowed gas exchange between the room and the arena resulting in an equilibrated low RH incubation environment. This was obvious from the absence of condensed free water drops on the sides of the arena. Also honeydew drops evaporated in a few minutes after excretion by the aphids. A high RH environment was created by sealing the Petri dish (arena) with parafilm. Free water drops were formed on the side of the dish after 24h incubation. Also honeydew drops did not evaporate once excreted. Two holes on the parafilm allowed enough gas exchange for the aphids without affecting the high RH, which was mainly maintained by the presence of the water agar layer and the transpiration of the leaf disk.

Computer tracking of aphids

Aphid clone R1 was used for these experiments. All experiments were carried out at high RH conditions achieved as described above. Also simultaneous (dual) dark phase filming was employed for these experiments (see relevant sections in Chapter 2).

4.2.2.2 Reproductive rate

The experiment was carried out using aphid clone R3. The method followed for the untreated (control) experiment is described in section 4.2.1.2. For the treated (infected) experiment the aphid was sprayed *in situ* in the individual Petri dish with 1.5ml of 10^6sp/ml *V. lecanii* (Vertalec) spore suspension. The dish was sealed with parafilm to ensure high RH and development of the mycosis.

4.3 Results

4.3.1 Comparative experiments between aphid clones

4.3.1.1 Behaviour analysis by visual observations

5 aphids were introduced onto a pepper leaf embedded on water agar in a 5-cm Petri dish (Figure 4.1). The dish was placed upside down on the camera stand (see Chapter 2) and the aphids were recorded for 5 or 7 days. Clones S and R3 were tested. Both mobility and honeydew production were studied. The tapes were analysed visually using the Observer computer programme to record the type of events and the time that they occurred. Every time that an aphid moved from its position or produced a honeydew drop these behaviours were recorded as events.

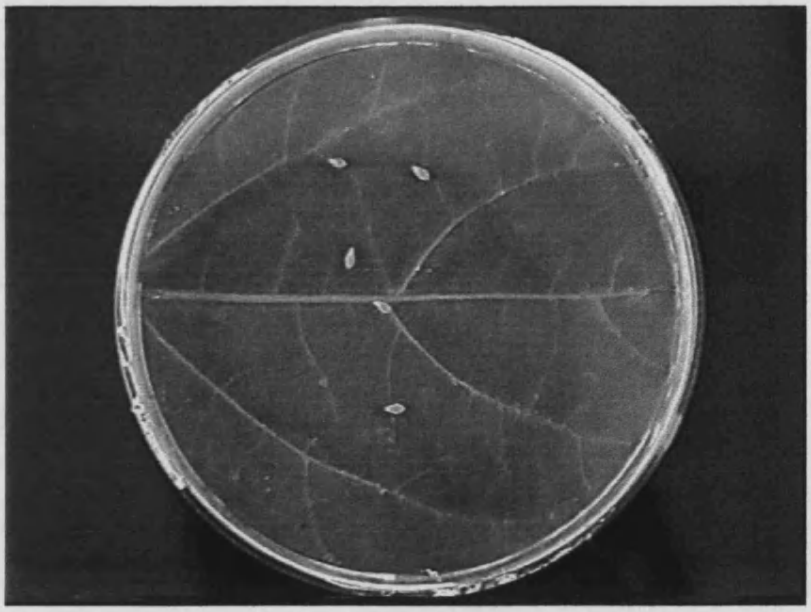


Figure 4.1 Filming arena (5-cm Petri dish) with 5 adult aphids (original image, captured from videotape)

The analyses of the mobility of the two aphid clones over a 5-day filming period are displayed in Figure 4.2. There is a distinct similarly between the behavioural patterns of the aphid clones. Day 1 is the day that the aphids were introduced to the leaf surface of the Petri dish. The aphids demonstrated immediately a searching behaviour for a suitable feeding site. However once settled the aphids were reluctant to move. Both clones showed less that 1 move per day per adult for the next two days (2 and 3). On day 4 for clone R3, the conditions in the filming arena became overcrowded and the adults were continuously disturbed by restless offspring, which were unable to find a free feeding place. This increased the observed mobility dramatically. On Day 5 observation was impossible for clone R3. For clone S the conditions in the filming arena continued to be optimal for days 4 and 5.

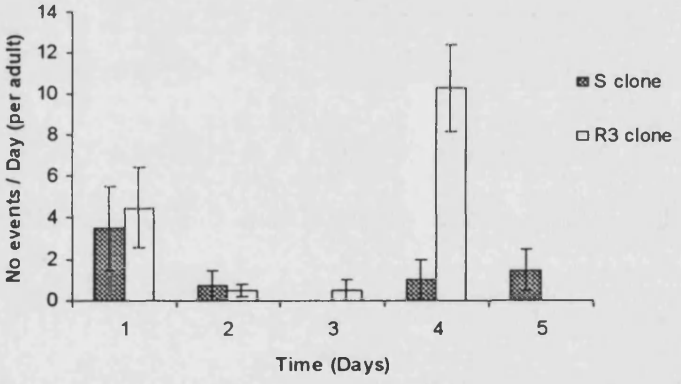


Figure 4.2 Aphid mobility during a five day incubation for clones S and R3

The activities of 4 aphids were filmed for 5 days continuously. The tapes were analysed visually. Every time that an aphid moved from its position, it was recorded as one event. The results are illustrated on the graph. The columns represent the arithmetic mean and the error bars the SEM. For clone R3 on Day 4 extremely crowded conditions were observed because of the presence of offspring. On Day 5 analysis was impossible for that clone.

For clone R3 the experiment was repeated, this time with a 7-day duration. On Day 4 the offspring were removed from the filming arena to avoid the problem that occurred in the previous experiment. The results are illustrated in Figure 4.3. The pattern is similar to the previous experiment. 3-4 mobility events per adult on Day 1 and 1 event per adult on the following days. The adults were again disturbed by the offspring on Day 4 increasing their mobility (6 events per adult). However, when the offspring were removed from the filming arena the mobility dropped to usual levels. On day 7 the mobility started to increase again. This time apart from the slow reestablishment of crowded conditions, dropping leaf quality was also involved. After a 7-day incubation, there were sites on the leaf with visible signs of deterioration.

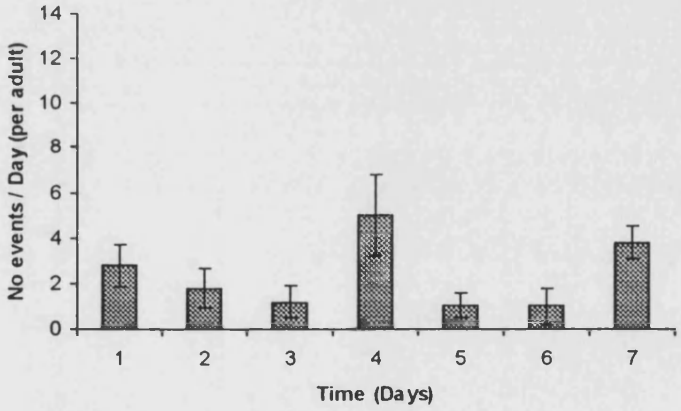


Figure 4.3 Mobility for aphid clone R3 during a 7-day incubation period

The movement events were recorded as described in the previous experiment. The columns represent the mean number of events (n=5) and the error bars the SEM. The adults were disturbed from the crowded conditions on day 4. The offspring were removed from the filming arena between day 4 to day 5.

Honeydew production was also investigated. When a honeydew drop was excreted the aphid body was placed in a characteristic erect position. When eventually the drop was released the aphid moved back to the original position. The honeydew drop itself was also visible if it landed on the surface of the protective filter of the camera lens. The number of honeydew drops excreted by each aphid was counted. The results for clones S and R3 are illustrated in Figure 4.4. The honeydew production was low on Day 1 but increased to 15- 20 drops/day for both clones on Day 2. For clone S honeydew production was stable for Days 3 to 5 (12-14 drops/day) and a similar stability was observed for mobility (from 0 to 3 move/day). For clone R3 the honeydew production was stable for days 2 and 3 while mobility was low. On day 4 increased mobility corresponded with reduced excretion of honeydew droplets. Clearly honeydew production was inversely related to the level of activity (mobility) of the aphids for both clones.

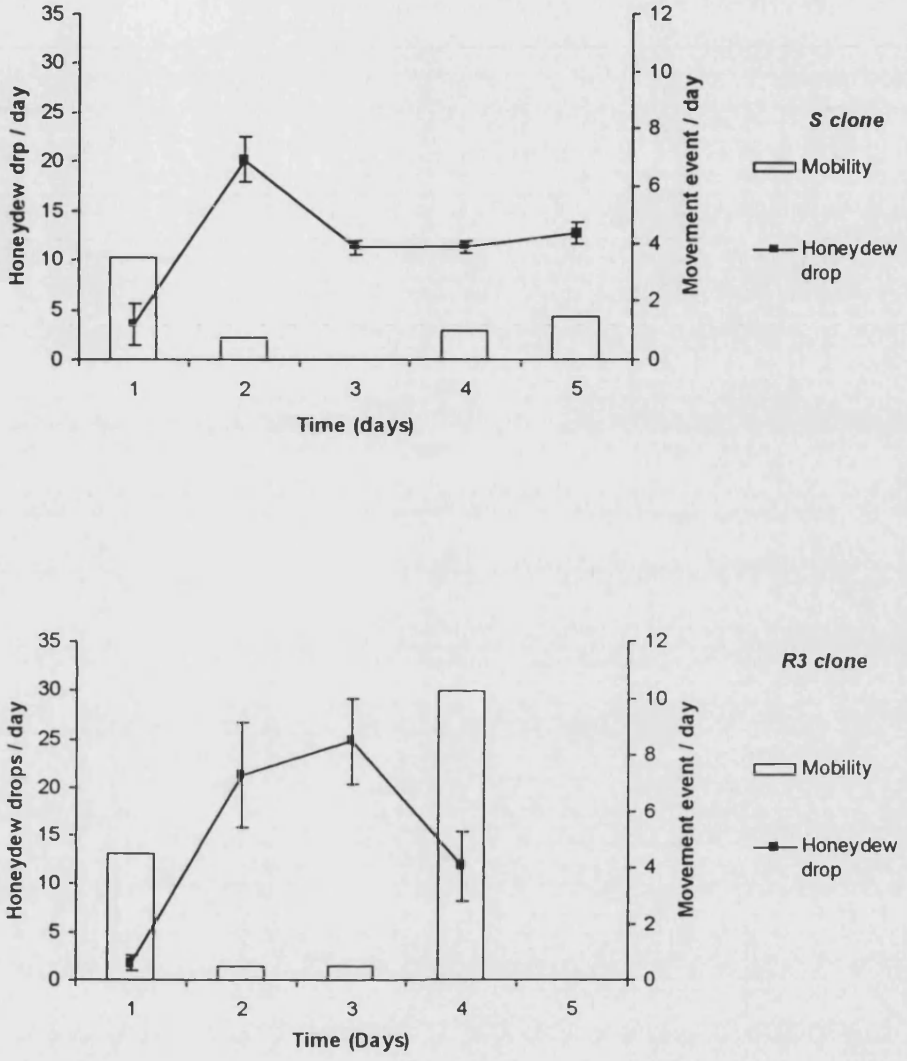


Figure 4.4 Honeydew production in relation to time and mobility, for clones S and R3

The production of honeydew droplets per day is illustrated on this graph. The points represent arithmetic mean (n=4) and the error bars the SEM. The mobility is represented with columns (arithmetic mean, n=4).

Preliminary experiments indicated that the interval between two honeydew drops was relatively consistent. The time intervals between two drops were retrieved from the experimental data for clones S and R3. The intervals were plotted according to their duration in Figure 4.5. The distribution curves showed distinctive peaks of 35-40 min for clone S and 25-30 min for clone R3. The two curves were similar and more than 70% of the observed interval durations ranged between 20 - 50 minutes for both clones.

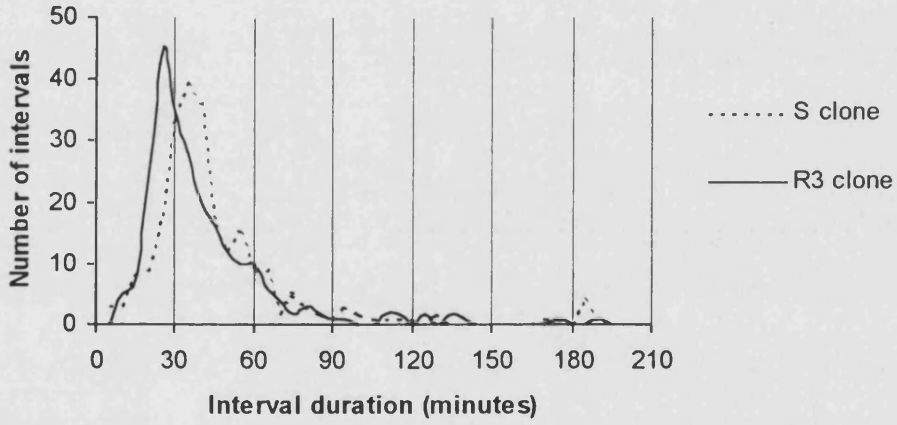


Figure 4.5 Distribution of interval duration between honeydew drops.

The intervals between successive honeydew drops from behavioural data from 4 observation days and 4 adult aphids are plotted against their frequency.

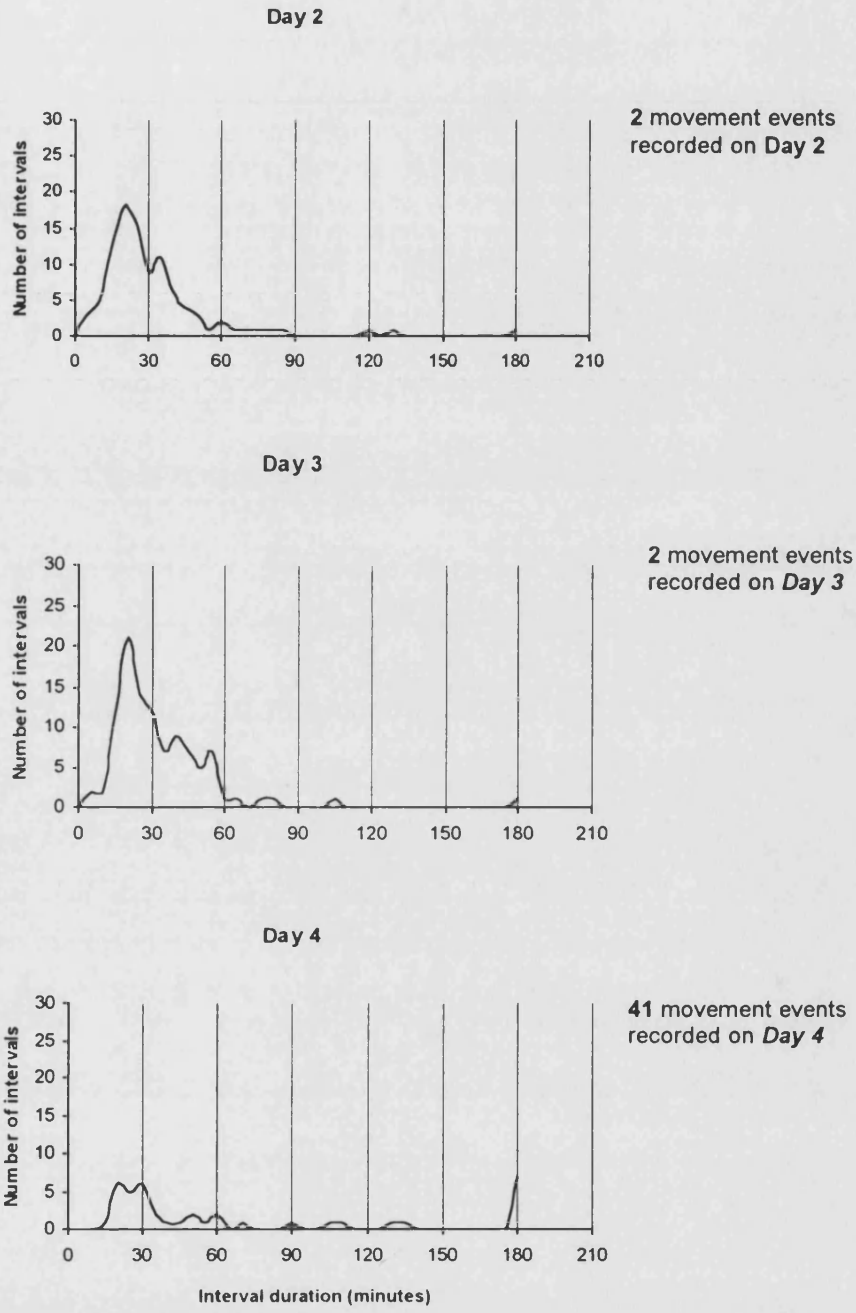


Figure 4.6 Effect of mobility on interval duration between honeydew drops for clone R3.

Interval duration between honeydew drops in relation to frequency is plotted on this graph (data from 4 adults) for days 2-4 of the experiment. The movement events on the side of each graph represent the total number of events that were recorded during that day from the same 4 adults. The data were retrieved from the 5-day experiment for aphid clone R3 (Figure 4.2).

Mobility affected the number of honeydew drops excreted (Figure 4.4). However rhythm of excretion was not markedly affected as shown in Figure 4.6. On days 2 and 3, where low mobility was observed (2 events/day) the peak of the curve of interval duration was between 20 - 25 minutes. On day 4 where high mobility occurred (41 events/day) there was still a peak (though less produced) between 20 and 25 minutes.

The consistency in the timing of excretion of honeydew drops illustrated in Figure 4.6 can be explained by observing in detail the behaviour of individual aphids (Figure 4.7). An aphid that is not mobile will excrete honeydew drops with a stable rate through out the day; in the case illustrated in Figure 4.7-*A* one drop every 15 -25 minutes. When an aphid moves the honeydew excretion stops but once the insect settles excretion continues again with the same rate (Figure 4.7-*B*). Even when an aphid has high feeding activity most of the honeydew drops will be excreted at intervals of 15- 25 minutes (Figure 4.7-*C*).

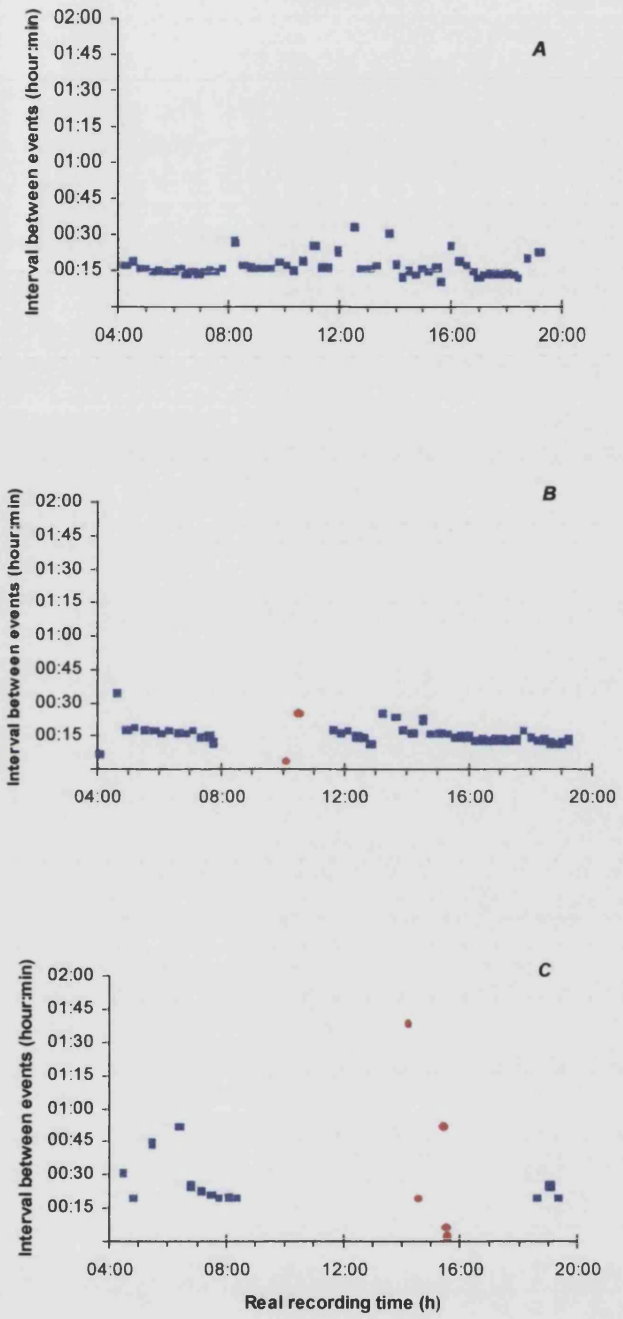


Figure 4.7 Effect of movement events on the excretion rate of honeydew drops for clone R3.

The data plotted in these graphs refer to individual aphids selected from set of results to illustrate the effects of movement events on the production of honeydew. The blue squares represent excretion events and the red dots movement events. The events are plotted on the *x*-axis in relation to the time that they occurred during the day. On the *y*-axis the events are plotted in relation to the time interval (hour:min) elapsed from the previous event (sequential events of excretion or movement). The data were retrieved from the 7-day experiment for clone R3 (Figure 4.3).

4.3.1.2 Behavioural analysis using the aphid tracking computer programme

5 aphids were introduced to a pepper leaf embedded on water agar in a 5-cm deep Petri. The dish was placed under the camera (see Chapter 2) and the aphid activities were recorded over 7 days. Only clone R1 was studied. The experiment was very much based on the previous set up. However the behavioural analysis was done using the aphid-tracking programme instead of visual observation and the set up was slightly modified to meet the needs of the computer programme. The programme was developed to calculate accurately the distance covered by the adults.

The distance covered by an aphid with time as calculated by the programme in a 4 hour long analysis set is displayed in Figure 4.8. When the aphid was mobile the distance covered was linearly related to the time elapsed. This suggests that the aphid travelled at a stable speed, which is expressed by the slope. The duration of each event can also be estimated from the graph as the start and end points can be identified. In Figure 4.9 the average distance covered by 5 aphids per day in a 7-day experiments is illustrated (16h of recording - 12h of analysed tape). The data from Day 1 (the day that the aphids were introduced to the arena) are not displayed, since in that period the aphids were allowed to settle. The average distance ranged from 45 to 210mm a day. The over all average was around 100mm a day.

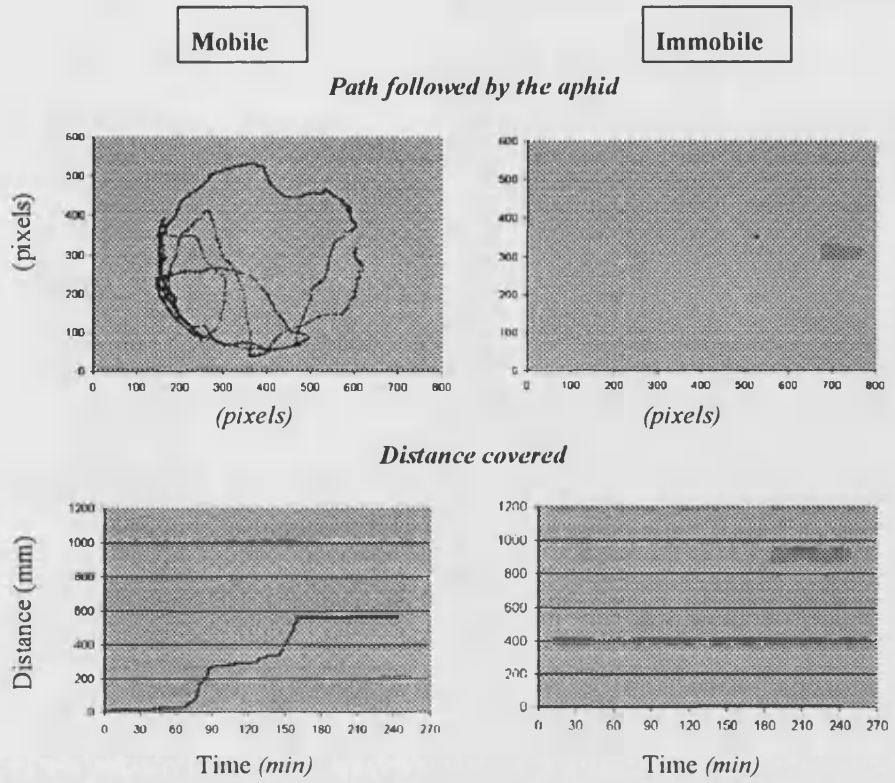


Figure 4.8 Cases of mobile and immobile aphids and the representation of the data after the analysis.

The path followed by one aphid in a 4-hour period is illustrated in the top graphs. For the immobile aphid the path is represented by a dot. The distance covered by that aphid with the time is illustrated in the lower graphs. In the case of the mobile aphid the distance covered was 580 mm, mainly in two activity bursts (events) that lasted around 15 min each.

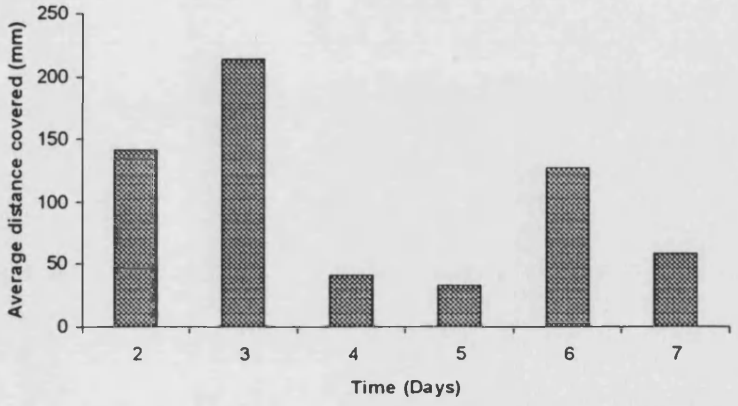


Figure 4.9 The mean distance covered per day by adult aphids (n=5) in a 7day experiment for clone R1

Distance was covered mainly during the few first days (Day 2 and 3) (see Figure 4.10). Highly active aphids (aphid 1 and 4) covered just over a meter (1000mm) in total during the 7-day period. Extremely inactive aphids could practically be considered immobile; aphid 5 covered just over 20 mm of distance in total during the whole experiment.

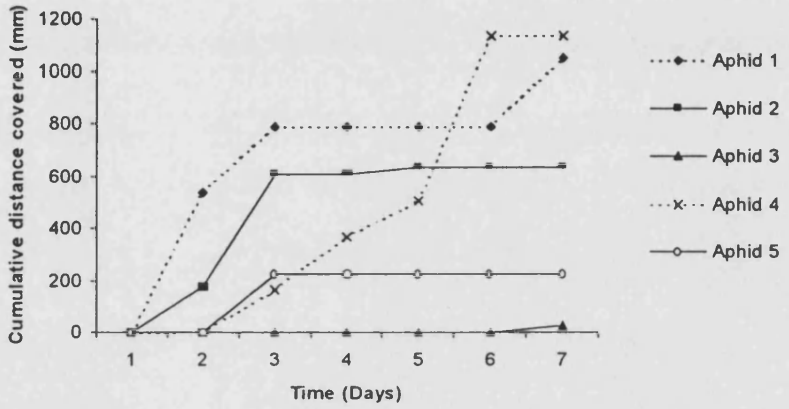


Figure 4.10 Cumulative distance covered with time by individual aphids during the 7day incubation period.

The tracking programme could also collect information about the surface area of a tracked object (Figure 4.11). Such information was of most value when the aphid was relatively immobile: it was possible to determine when the aphid was producing an honeydew drop or an offspring from an apparent temporary decrease in size. The timing of the honeydew excretion is remarkable, even when an offspring is born (Figure 4.11-*B* & *C*). However the area readings where very sensitive to many uncontrollable factors (shadows, position of the body in relation to the camera, the nearly presence of an other aphid etc.) Hence, this method of analysis could not continuously produce data on the aphid behaviour, under high complexity-filming arenas as these used in the particular experiments.

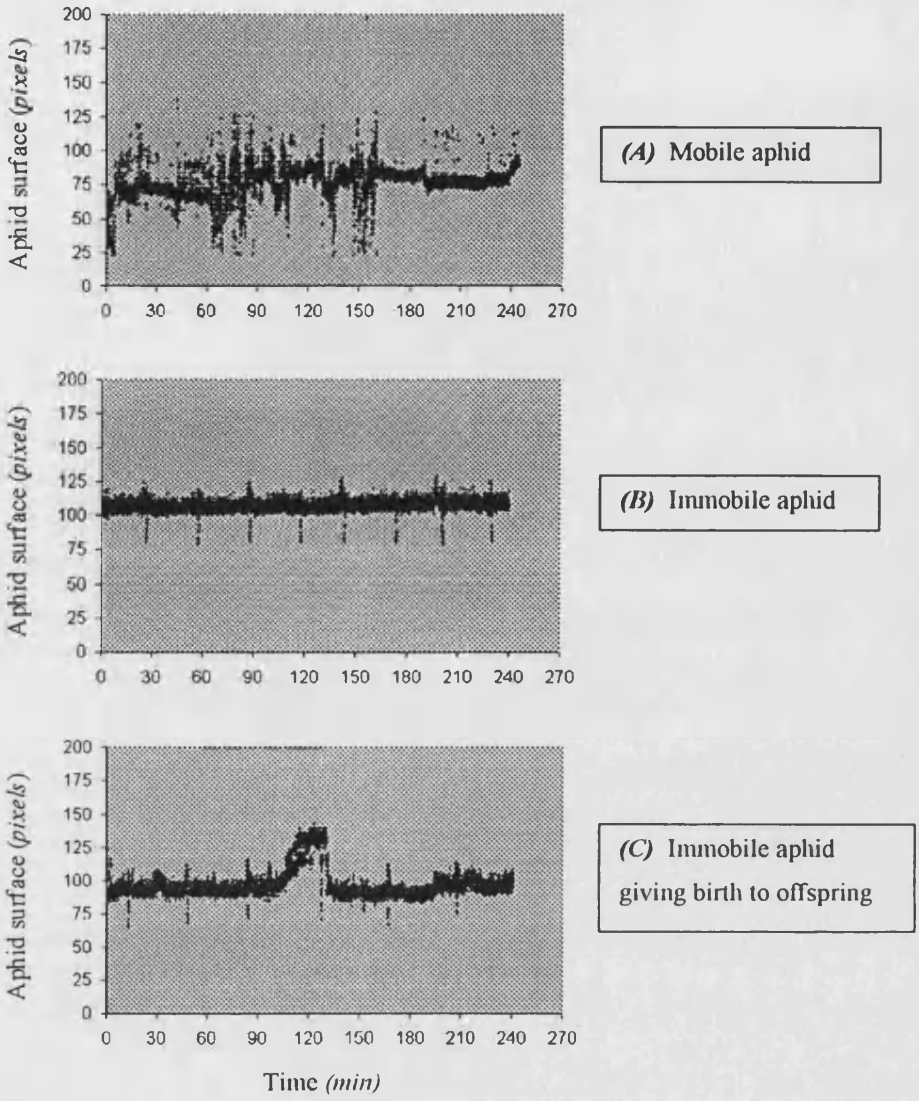


Figure 4.11 Surface area of the tracked aphid as recorded by the programme with the time in the case of mobile and immobile adults

(A) The surface of the aphid was changing continuously as the insect was moving producing high variability of the area tracked by the programme. The area was recorded in pixels.

(B) However when the aphid was immobile the surface of the aphid varied little and it was recorded between 100 and 120 pixels. When the aphids was excreting a honeydew drop the body was held erect, resulting in a reduction in aphid surface area. On the graph each event is represented by straight vertical line.

(C) During birth the surface of aphid was apparently increased when the young was emerging from the mother. On the graph this is represented as increase of size, reaching 150 pixels. As the offspring is detached from the adult the original size is immediately regained (100 pixels).

4.3.1.3 Reproductive rate

This experiment was designed to compare the reproductive ability of the aphid clones. Clone S was not included in the experiments due to problems retaining populations of this clone in culture in the lab. The number of offspring produced by 7 adults for each aphid clone (R1. R2 and R3) was recorded daily for 5 days. The average number of offspring born with time is shown in Figure 4.12. The data from all three clones showed a significant strong correlation with time ($P<0.01$) and fitted a simple linear model ($r^2>0.93$) (see Appendix 5). The average slope of the best-fit line represents the reproductive rate of each clone. ANOVA showed that reproductive rate between clones R1and R3 was not significantly different ($P<0.05$) (Figure 4.13). However clone R2 was significantly greater than the other two clones.

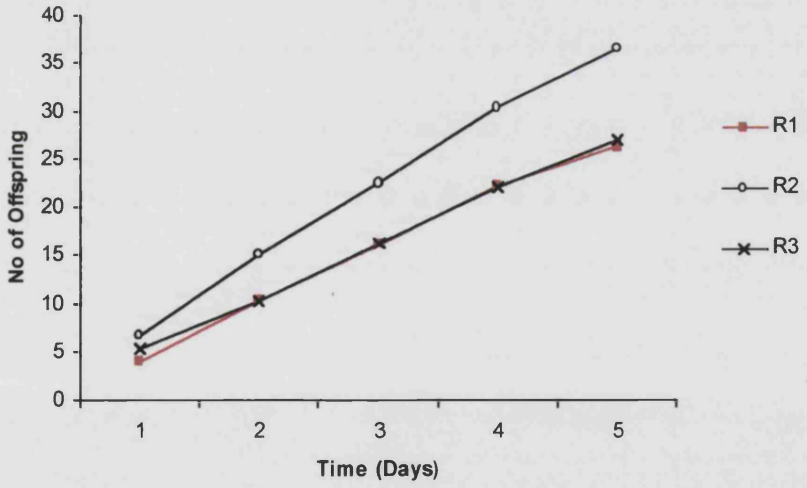


Figure 4.12 Cumulative number of offspring born with time for the aphid clones R1, R2 & R3

The number of offspring was counted daily. The points represent the arithmetic mean (n=7) of each count.

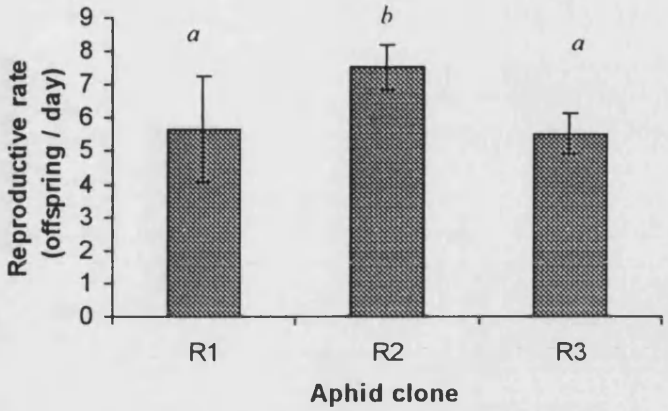


Figure 4.13 Reproductive rate for aphid clones R1, R2 & R3

The columns represent the arithmetic mean of the reproductive rate (slope) recorded for each aphid clone (n=7) and the error bars the standard deviation. The data were normally distributed and an ANOVA was applied. Columns marked with the same letter are not significantly different ($P<0.05$).

4.3.2 Effects of fungal infection on aphid behaviour

4.3.2.1 Behaviour analysis by visual observations

The behaviour of 5 aphids, (clone R3) on a pepper leaf disk embedded in water agar in a 5 cm Petri dish, was recorded for 7 days and analysed. The incubation conditions were a temperature of 24°C and photoperiod 16h L:8h D. In the case of low RH the conditions were similar to the room relative humidity (%) and for the high RH case the conditions were saturated (RH~100%).

Low RH experiment

The results from the analysis of the aphid mobility under low RH incubation conditions are displayed in Figure 4.14. Both treatments displayed a similar behavioural pattern over the time period. In general mobility was low, apart from the

first day (search for feeding site), and overcrowding disturbed the aphids, on day 4 for control and on day 5 for fungus-treated aphids. Offspring were removed from the control during the dark phase of day 4 and this caused the mobility to change back to a low level on day 5. Fungus-treated aphids did not develop any symptoms of mycosis throughout the experiment up to day 7. The offspring were removed from the filming arena (Petri dish), the dish was sealed with parafilm and was incubated for 3 more days with out filming the aphid behaviour. The first infected aphids were observed 2 days after the dish was sealed.

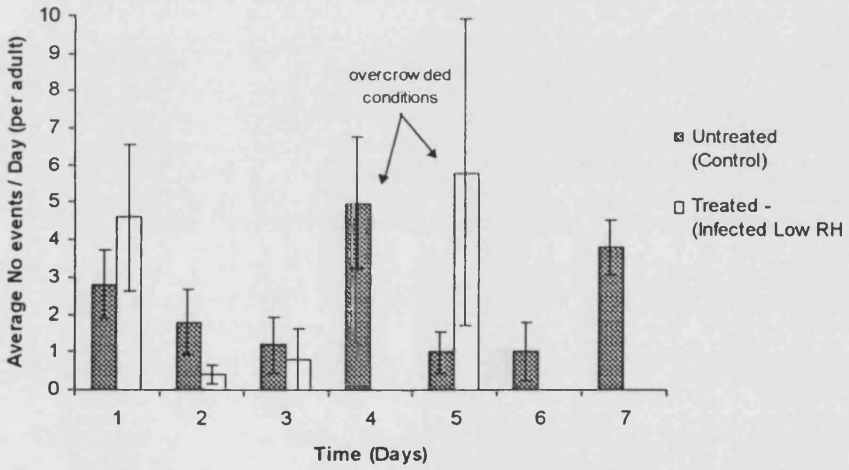


Figure 4.14 Mobility for fungus-treated and control aphids (clone R3) during a 7-day incubation period under low humidity conditions

The movement events were recorded continuously for 7 days. The columns represent the mean number of events from 5 aphids and the error bars the SEM. The adults were disturbed by crowding on day 4 for control treatment and the offspring were removed from the filming arena between day 4 to day 5. For infected treatment the aphids were disturbed by the offspring on day 5; the offspring were not removed during the experiment.

The honeydew production was similar in control and fungus-treated aphids and in both treatments it was inversely correlated with mobility (see Figure 4.15).

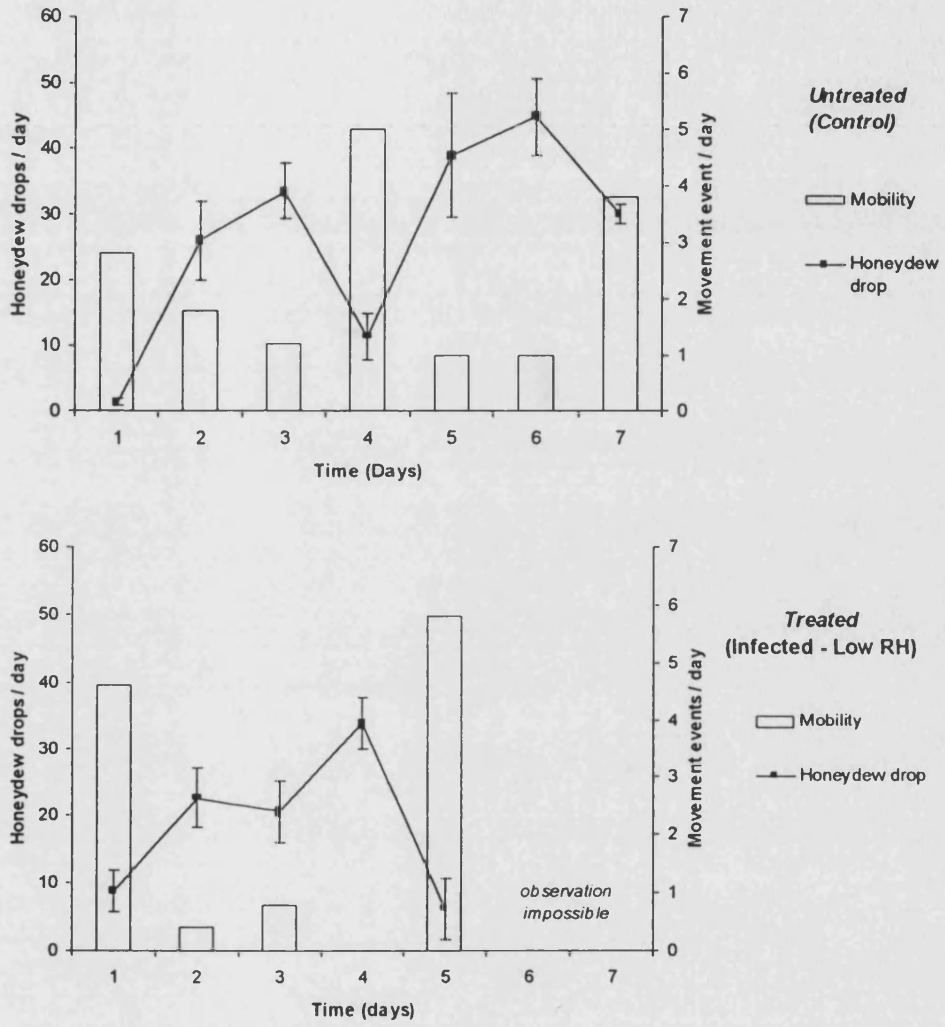


Figure 4.15 Honeydew drop excretion in relation to time and mobility for Untreated (control) and Treated (infected) aphids (clone R3) incubated for 7 days under low RH conditions.

The production of honeydew droplets per day is illustrated on this graph. The points represent the arithmetic mean (n=5) and the error bars the SEM. The mobility is represented with columns (arithmetic mean, n=5). In both cases increased mobility reduced the number of honeydew drops produced per day.

High RH experiment

The mobility of fungus-treated and control aphids incubated under high RH conditions over a 7-day filming period was recorded. The number of movement events per day varied for both treatments during the course of the experiment. For control aphids this was due to overcrowding. Fungus infected aphids, however, were not encouraged to move by environmental factors. To compare the pulled data from the two treatments Chi-square test was used (Table 4.1). On day 3 significant difference was recorded between mobility of mycosed and control aphids. On day 6 two mycosed aphids were dead and on day 7 all 5 aphids died resulting in the absence of movement events.

Table 4.1 Total number of mobility events recorded per day for treated and untreated aphids (clone R3) during a 7-day incubation period under high humidity conditions

	Total number of mobility events recorded per day						
	Day 1	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Day 7*
Untreated (Control)	14	9	6	* 25	5	5	19
Treated (Vertalec)	7	15	27	0	10	9	** 0
X^2	2.3	1.5	13.4	25.0	1.7	1.1	19.0
Probability	*n.s.*	*n.s.*	*<0.05*	-	*n.s.*	*n.s.*	-

The movement events were recorded continuously for 7 days. The numbers represent the pooled number of events from 5 aphids. For infected treatment the aphids were not disturbed at any time. Chi-square test was applied on the data for each day. The high mobility observed on Day 3 for the treated (infected) aphids was significantly different from the control (X_t^2=3.84 for *d.f.*=1 and P<0.05, *n.s.* for 'not significant').The (*) on day 4 indicated that the adults were disturbed from the crowded conditions for control treatment and the offspring were removed from the filming arena between day 4 to day 5. The (**) on Day 7 indicates that the treated aphids were dead.

Honeydew production is recorded alongside mobility in Figure 4.16. In controls honeydew production is maintained at a high level as long as mobility is low. This relationship between exertion and movement is not so clear in mycosed insects, where there is a decline in honeydew production from day 3 through to the death of the aphids on day 7.

To understand better what happened during those 7 days a more detailed analysis of individual behaviours is presented in Figure 4.17. All adults were active and were recorded to move numerous times within the period from day 1 to day 3. However 3 aphids (aphid N° 2, 3 and 5, all indicated by blue coloured lines) did not move after that day. These aphids also showed earlier reduction of honeydew excretion compared to aphids that were mobile through out the experiment (No 1 and 4, indicated by red line). In Figure 4.18 the levels of honeydew drop production and the rate that they were excreted is displayed for the same individual adult aphids.

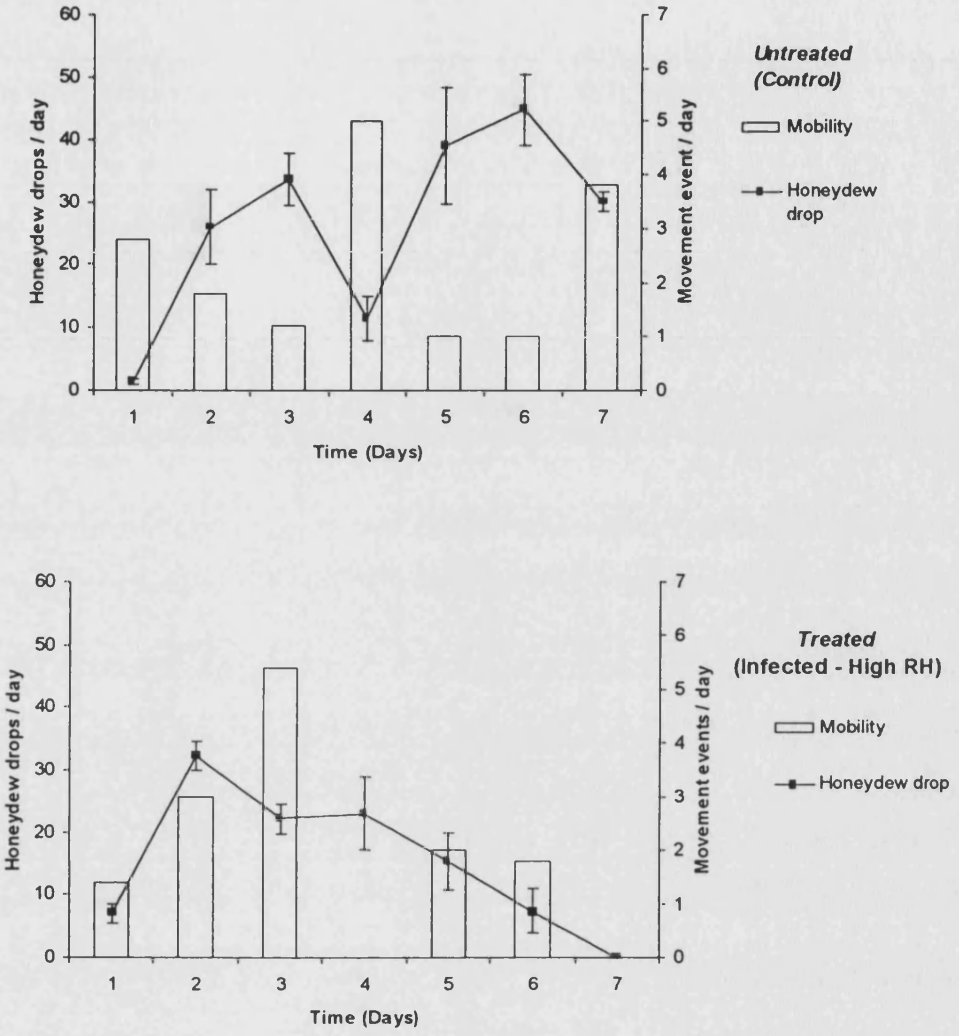


Figure 4.16 Honeydew excretion in relation to time and mobility for fungus-treated (infected) and control aphids (clone R3) incubated for 7 days under high RH conditions.

The production of honeydew droplets per day is illustrated on this graph. The points represent the arithmetic mean (n=5) and the error bars the SEM. The mobility is represented with columns (arithmetic mean, n=5).

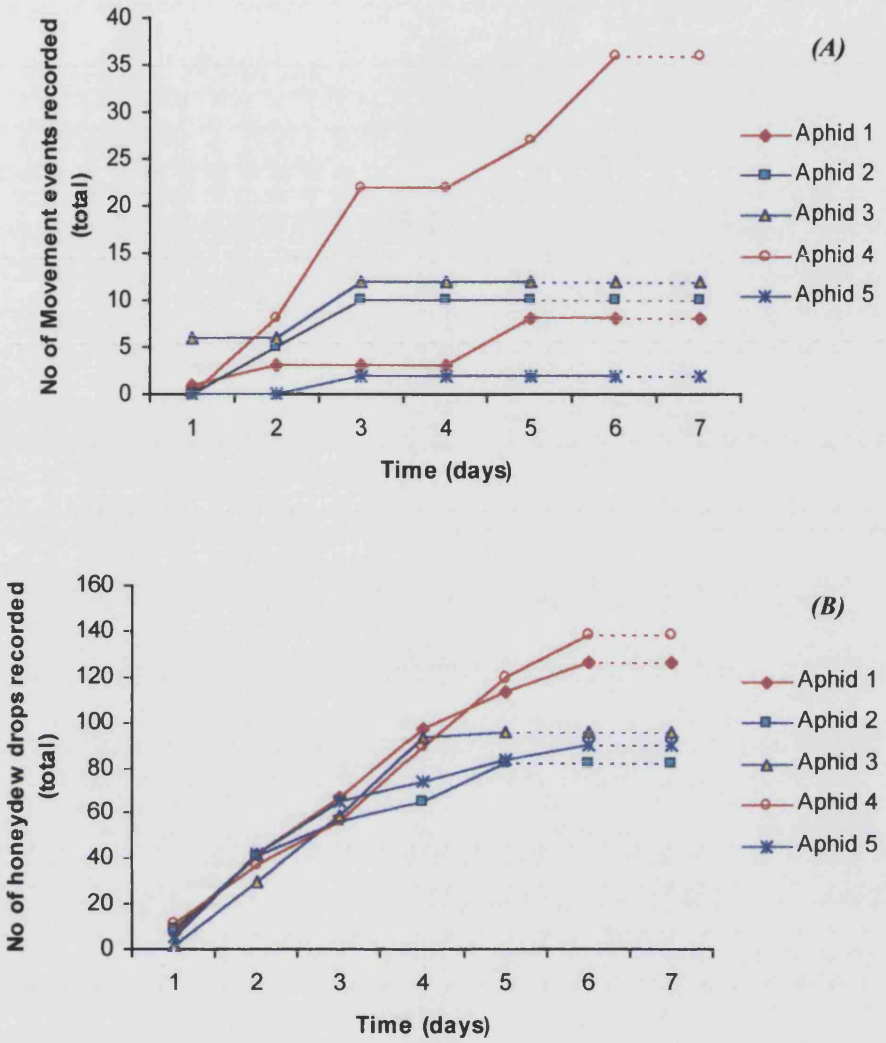


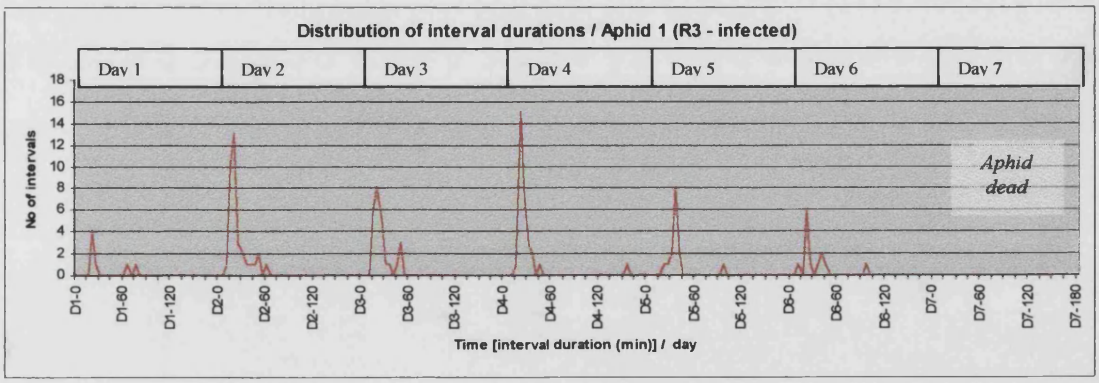
Figure 4.17 Number of movement events (*A*) and honeydew drop (*B*) recorded for individual adult aphids with time.

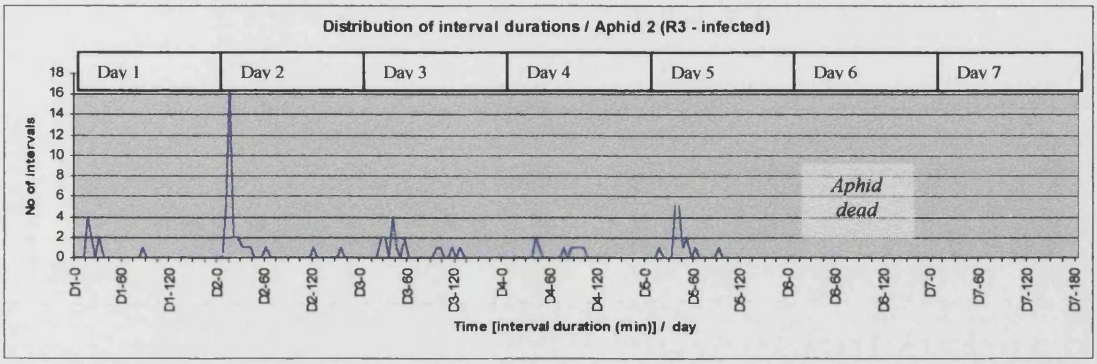
Aphids that were recorded to be inactive after day 3 (relatively early in the experiment) are indicated by blue coloured lines (*A*). These individuals also stopped excreting honeydew earlier in the experiment (*B*) compared to the active adults (red line) that produced honeydew until day 6. Dotted line indicates aphid death.

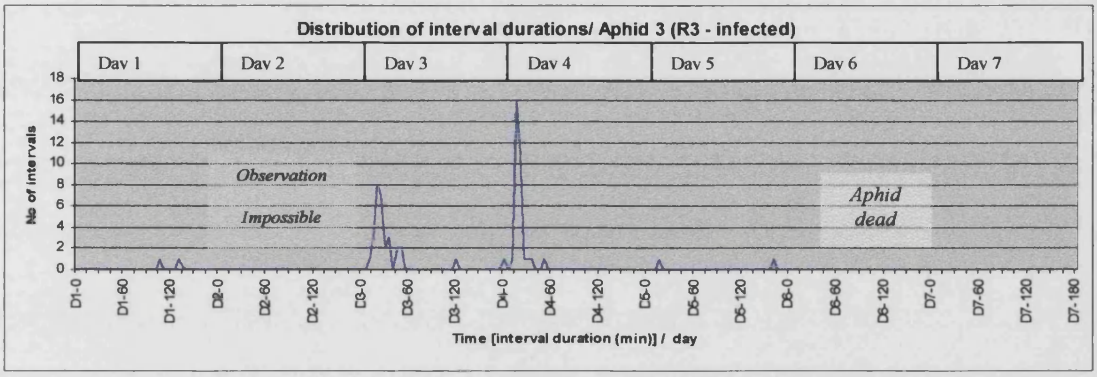
Figure 4.18 Distribution of interval duration between two honeydew drop events from individual adult aphids (clone R3) from the fungus-treated (infected) experiment incubated for 7 days at high RH.

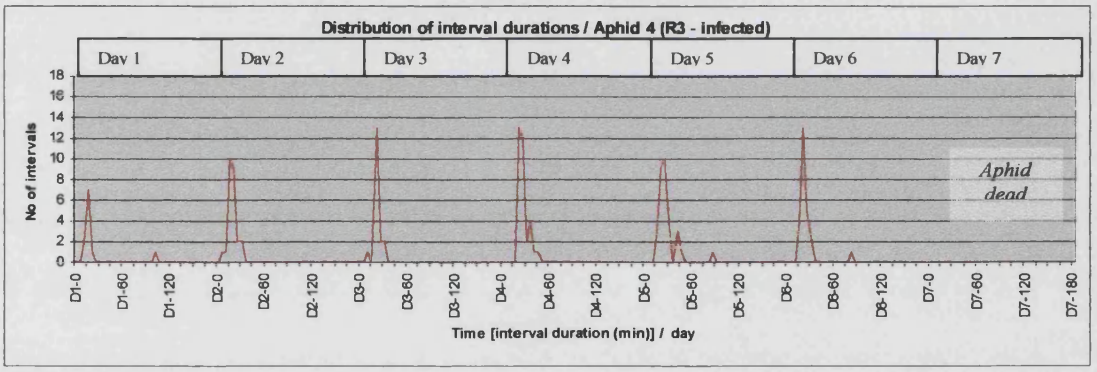
Y axis: number of intervals with a specific duration per day. *X* axis: the data from 7 days (D1-D7) are plotted. The beginning of the day is marked as D1-0, and the end as D1-120, The next day follows in sequence (D2-0, etc.) For each day the number of intervals with a specific duration are plotted against their duration (between 0 to 120 min, split in 24 5-minute ranks (from 5 to 120 minutes)).

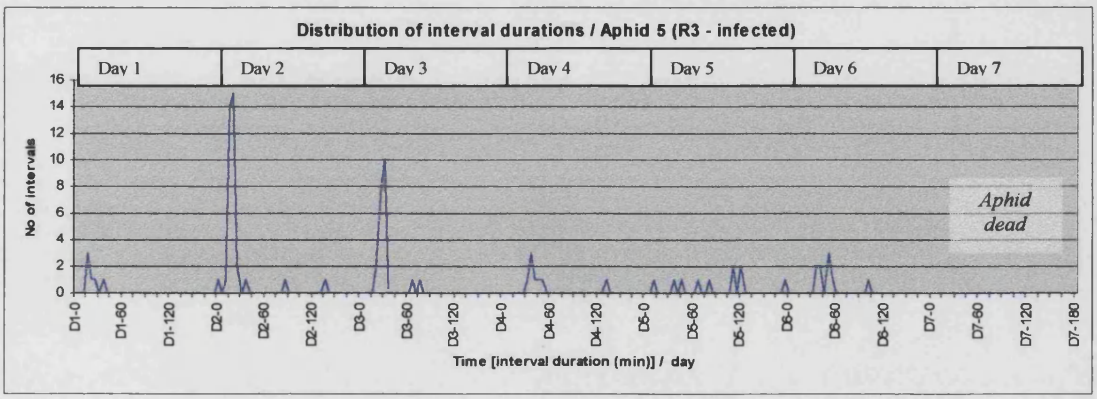
- Aphid 1 and aphid 4 (in red lines) showed clear peaks at 30min of interval between honeydew drops from day 1 to day 6.
- Aphid 2 lost it ability to excrete honeydew drops with a stable rate on day 2. However, it produced honeydew in low amounts until day 5
- Aphid 3 stopped producing honeydew with a stable rate on day 4 and the excretion ceased practically after that day.
- Aphid 5 stopped producing honeydew with a stable rate on day 3. It kept on excreting honeydew at low amounts until day 6, in a similar pattern to aphid 2.




Distribution of interval durations / Aphid 2 (R3 - infected)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
No of intervals
Aphid dead
Time [interval duration (min)] / day


Distribution of interval durations/ Aphid 3 (R3 - infected)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
No of intervals
Observation Impossible
Aphid dead
Time [interval duration (min)] / day


Distribution of interval durations / Aphid 4 (R3 - infected)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
No of intervals
Aphid dead
Time [interval duration (min)] / day


Distribution of interval durations / Aphid 5 (R3 - infected)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
No of intervals
Aphid dead
Time [interval duration (min)] / day

The level of honeydew production followed one of two patterns (see Figure 4.18):

a) Production dropped from high to low levels within a day, where it was maintained for a few days until it ceased and the insect died (indicated by sporulation) (aphids 2, 3 and 5 / blue lines) or,

b) Honeydew was excreted continuously at a normal high level, until it stopped suddenly at the point of death, sporulation occurred the following day (aphids1 and 4 / red lines).

Aphids 1 and 4 exhibited a pattern of movement on days 5 and 6 that was distinct from that observed early in the experiment (days 1-3) by the infected aphids and during control experiments. These movement events were non-directional and slow and were carried out, while the insect was shaking. After the end of a period of such behaviour the insect did not move again and soon the honey dew excretion ceased. These events were characterised as pre-death behaviour.

In summary aphids tended to be mobile during the first 3 days of mycosis and produce large amounts of honeydew. After this initial period two different patterns emerged. For aphids 2, 3 and 5, there was a sudden early reduction of honeydew production combined with immobility. These aphids in comparison with the insects from the other group died earlier. The pattern followed by aphids 1 and 4 showed high production of honeydew and relatively normal mobility through out the experiment. Sudden halt of all function and cadaver sporulation was observed sometime after a characteristic pre-death mobility period.

4.3.2.2 Behavioural analysis by aphid tracking commuter programme

All experiments used the same basic filming and analysis methods. In all the cases the filming started 24h after application of the spore suspension (or sterile dH_2O for untreated) to allow settling of the insects. The temperature was set at 24°C, photoperiod at 16h L: 8h D and RH was high through out the experiment (~100%).

The first set of trials involved 7 day filming of treated (infected) and untreated aphids under high humidity conditions. Filming lasted either 16h per day (12h of total time analysed) or 23h, with implementation of dark phase filming (18h of total time analysed). The second set of experiments involved simultaneous filming for 23 h per day (dual camera set up and dark phase filming, details in relevant section of Chapter 2) of treated and untreated insects, for three days. *M. persicae* clone used was R1 and the *V. lecanii* isolates Vertalec and KV42 were applied (1.5 ml of 10^6sp/ml spore suspension).

<u>7 day experiments (without dark phase filming)</u>

The filming of the aphids started after a 24h incubation (from day 2 onwards) and lasted for 6 days (until day 7). The isolate applied was Vertalec. The results from the computer analysis of the aphid behaviour are displayed in Figure 4.19.

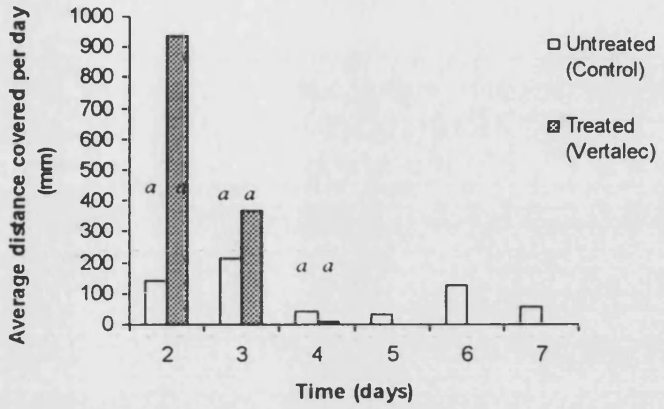


Figure 4.19 Average distance covered per day by treated (Vertalec) and untreated aphids (R1) with the time (12h of videotape analysis/day).

The columns represent the arithmetic mean (n=5). The data are not naturaly distributed and the Mann Whitney *U* test was applied for statistical analysis between the treatments. Columns of the same day marked with the same letter are not significantly different ($P<0.05$).

Untreated aphids covered an average daily distance ranging between 50 to 200mm through out the experiment (Figure 4.19). On the other hand treated aphids covered most of the distance on days 2 and 3. Their activity ceased from day 4 onwards. Death of treated aphids by fungal infection was estimated between day 4 and 5, two days after the activity peak was observed (on day 2). The treatments were not significantly different on days 2, 3 or 4, because there was large within treatment variation (Figure 4.19).

In some cases cessation of honeydew production was noted. This event that could be defined as 'aphid death' (see Figure 4.20). After death the aphid body became darker in colour (Figure 4.21), and with in a few hours hyphae started emerging and soon covered the cadaver

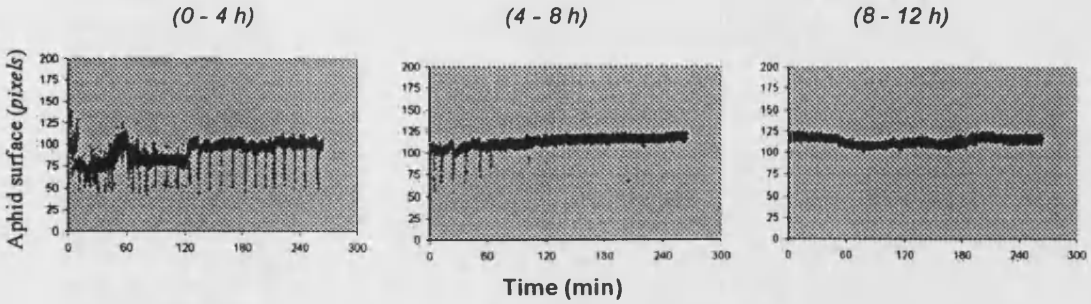


Figure 4.20 Observing the sudden halt of excretion of honeydew drops from the data collected by the tracking programme for the surface of the insect.

The data were collected from the 4th day of incubation. The surface of an aphid is plotted against time in three sequential sections, 4h long each (12h total). The vertical lines denoted honeydew production. In the first section (0-4) the aphid is excreting honeydew at a high rate. In the second section (4-8) the excretion suddenly stops, followed by a period of complete inactivity, which is continued in the last section (8-12).

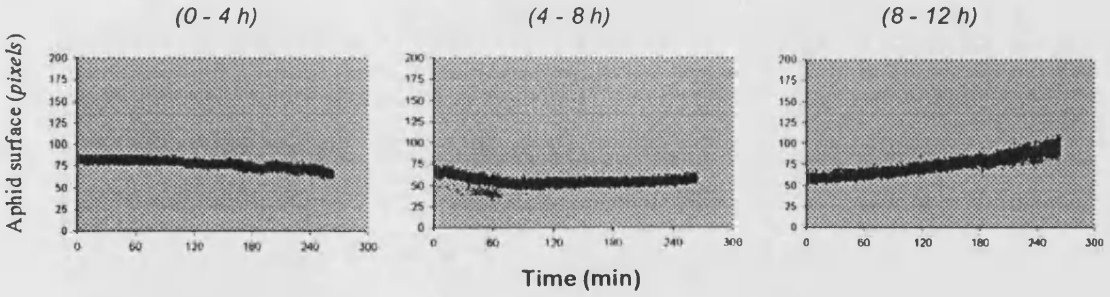


Figure 4.21 Alterations of aphid surface after the death of the infected insect as recorded by the tracking programme.

The data were collected from the 5th day of incubation. The surface of an aphid is plotted against the time in three sequential sections, 4h each (12h total). In this case the programme records the colour alteration of the dead aphid, while the actual aphid size remains stable. After death the body colour tone of the infected insects was observed to become progressively darker. As the programme was recording the number of pixels of that selected area (pixels selected on their threshold value ('brightens')) this resulted in a reduction of body surface area (see: end of first section (0-4) until the middle of second section (4-8)). The fungal hyphae soon started to emerge from the cadaver, forming the typical covering of white mould. The colour tone of the dead aphid again began to alter, and that was recorded as an increase of the selected surface (section three (8-12)).

7 day experiments with dark phase filming

The experiment was repeated, this time filming occurred during both light and dark phases of the photoperiod. This allowed a more complete description of the behaviour responses of the aphids. The results are illustrated in Figure 4.22. The distance covered per day by control aphids ranged from 50 to 250mm. The distance/day for the treated aphids ranged from 50 to 300mm until incubation day 5, while movement was absent for days 6 and 7. Aphid death occurred between days 5 and 6. The average distance covered, per day, was not significantly different between the two treatments ($P<0.05$). For treated aphids, day 3 was the most active day, 2 days before aphid death.

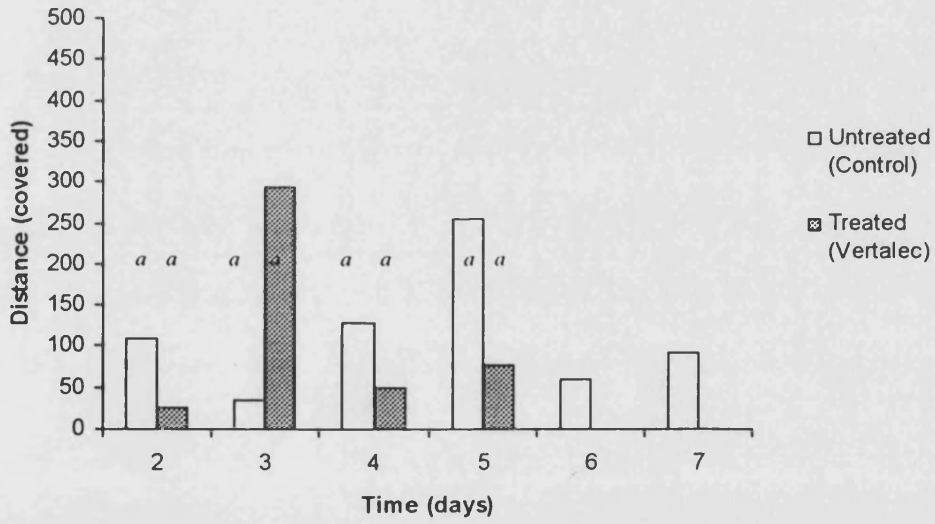


Figure 4.22 Average distance covered per day by treated (Vertalec) and untreated aphids (R1) with time (18h of videotape analysis/day).

The columns represent the arethmetic mean (n=5). The data are not naturaly distributed and the Mann Whitney *U* test was applied for statistical analysis between the treatments Columns of the same day marked with the same letter are not significantly different ($P<0.05$).

The above experiment was repeated twice using the *V. lecanii* isolate KV42. The results are illustrated in Figure 4.23. Treated aphids showed activity only on days 2 and 3. In both trials the mobility peak was on day 2. The average distance covered on day 2 by treated insects in the second trial (KV42-2) was significantly greater than that covered by untreated insects (Figure 4.23). In all the other cases distance covered per day between treated and untreated insects were not significantly different. Aphid death was estimated between days 4 and 5, two days after the mobility peak was observed.

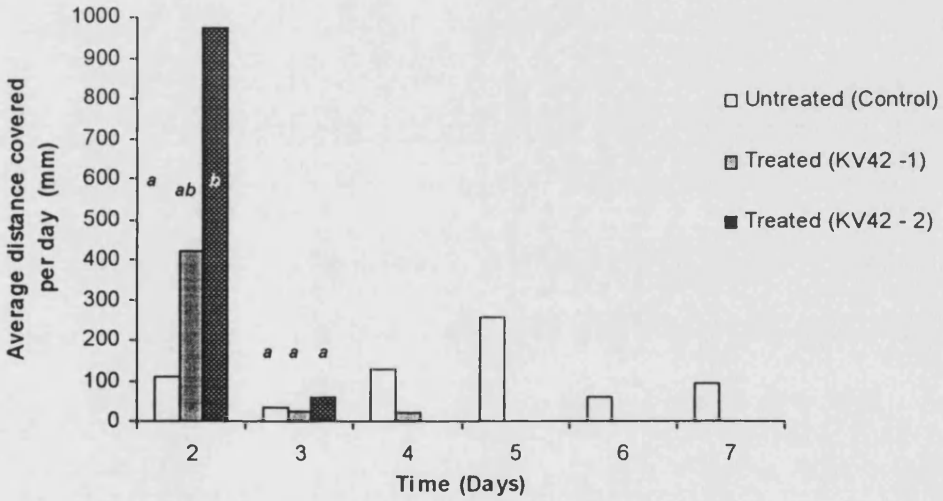


Figure 4.23 Average distance covered per day by treated (KV42) and untreated aphids (R1) with the time.

The columns represent the arithmetic mean (n=5). The data are not naturaly distributed and the Mann Whitney *U* test was applied for statistical analysis between the treatments. Columns of the same day marked with the same letter are not significantly different ($P<0.05$).

3 day experiments with simultaneous (dual) and dark phase filming

The experiments were repeated using both fungal isolates (Vertalec and KN42) on aphid clone R1 for shorter time periods (4 days). Analysis was focused on the first days of the development of the mycosis. In addition to the dark phase filming, both treated and untreated insects were filmed simultaneously (dual filming: see Chapter 2) to reduce variability from environmental factors.

The data from the tape analysis are displayed in Table 4.2. The mean distance covered by Vertalec and KV42 treated aphids was always longer than that covered by their respective controls on days 2 and 3.

Data were statistically analysed using a randomised block method. This method has been used to compare the effects of treatments done on different fields (blocks). In this case the blocks are the time periods that filming occurred. Randomised block method takes into account the fact that the experiments were done at different time periods, but that the treatments of each experiment were done simultaneously. Between-experiments variation was accounted for by subtracting the block effect (Sokal and Rohlf, 1995). The square root transformation stabilised the variation of the data between the experiments, which is required by the test (Fowler *et al.*, 1998; Sokal and Rohlf, 1995).

The results from the statistical analysis are displayed in Table 4.3. Vertalec treated aphids walked significantly more on days 2 and 3 than untreated aphids ($P=0.008$ and $P=0.050$ respectively). KV42 treated aphids also walked significantly greater distances than controls, but only on day 2 ($P<0.001$).

Table 4.2 Average distance covered per day (n=5) by untreated adult aphids and aphids treated with *V. lecanii* isolates Vertalec and KV42.

Vertalec Isolate

Experiment		Day 2	Day 3	Day 4
1	Untreated	2141.1	90.5	54.7
	Treated	4056.0	733.6	55.7
2	Untreated	353.8	61.3	218.4
	Treated	11479.2	959.6	0.0
3	Untreated	364.5	488.0	297.6
	Treated	664.3	840.3	249.5

KV 42 Isolate

Experiment		Day 2	Day 3	Day 4
1	Untreated	8.0	118.8	304.4
	Treated	301.8	136.9	0.0
2	Untreated	533.7	156.8	240.4
	Treated	2243.9	290.3	19.9

The experiments lasted 4 days. There are no data for day 1. Data extracted from 16 h of analysed tape per day (23 h filmed total/day). The two treatments for each experiment were filmed simultaneously.

Table 4.3 Statistical analysis of the data on distance covered from Table 4.2 using randomised block test on Genstat.

		Day 2	Day 3	Day 4
Vertalec Isolate	Untreated	*	*	*
	Treated	**	**	*
KV 42 Isolate	Untreated	*	*	*
	Treated	**	*	*

Square root transformation was used to stabilise the variance between the treatments. Treatments with the same number of (*) within a column are not significantly different ($P<0.05$).

4.3.2.3 Effects of mycosis on reproductive rate

The number of offspring produced by 7 adults for each treatment (control and Vertalec infected) was recorded daily for 6 days. The results are illustrated in Figure 4.24. For untreated aphids the number of offspring born per day (reproductive rate) ranged from 5.0 - 6.0 though out the experiment. For treated aphids the reproductive rate was stable in the first 4 incubation days, between 4.0 and 5.7 offsp./day and was not significantly different from the control (Table 4.4). Reproductive rate was reduced significantly on days 5 and 6. Aphid death occurred between days 6 and 7.

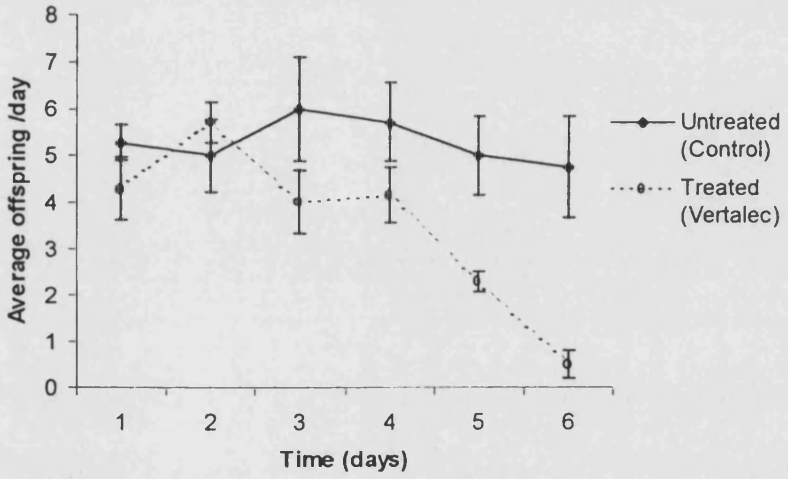


Figure 4.24 Average number of offspring born per day (reproductive rate) with time for untreated and treated (Vertalec) adults (aphid clone R3).

The points represent the arithmetic mean (n=7) and the error bars the SEM.

Table 4.4 Total number of offspring born per by treated and untreated aphids (R3) with the time

	Total number of offspring born per day					
	Day 1	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*
Untreated (Control)	37	35	42	40	35	19
Treated (Vertalec)	30	40	28	29	16	2
X^2	0.7	0.3	2.8	1.8	7.1	13.8
Probability	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*<0.05*	*<0.05*

The offspring production was recorded for 6 days. The numbers represent the pooled data from 7 aphids (4 aphids on day 6). Chi-square test was applied on the data for each day. Significantly different offspring production between treated and untreated aphids was observed on Days 5 and 6 (X_t^2=3.84 for *d.f.*=1 and P<0.05, *n.s.* for 'not significant).

4.4 Discussion

4.4.1 Behaviour of insecticide resistant and susceptible clones of *M. persicae*

Aphid behaviour was studied using visual analysis of 7-day videotape recordings for clones S and R3. Both clones showed a similar activity pattern with time. Once introduced to the leaf surface aphids explored the area in search of a feeding place, which is illustrated by the number of times that they were observed to change position on the arena (movement events). These results are in agreement with Klingauf (1987b) who describes the typical searching behaviour of aphids as sequential periods of waking and probing. Aphids need time to accept a new host and the searching behaviour can last up to several hours (Klingauf, 1987a; Schnorbach, 1983).

Adults of both clones were reluctant to move from their position under optimal conditions resulting in an average mobility of less than 1 event per adult per day. In some cases, individual aphids stayed immobile for 2 or 3 days. Preliminary experiments on whole plants indicated even less aphid mobility. However, in these experiments only the alteration of feeding site was recorded. Nevertheless, despite the low sampling rate (3 readings/day), the results were considered representative because aphid activity is also very low. Only 1 aphid out of 3 was found to move from its feeding site per day. These findings were also supported by experiments using the computerised tracking of R1 adults. The average distance covered by an aphid during a day would be around 4 to 20 cm.

Aphids have been observed to feed continuously and stop only when moving to a new location or when moulting (Klingauf, 1987a). Leszczynski *et al.* (1995) using EPG estimated that the grain aphids (*Sitobion avenae* F.) spent 80% the total time ingesting sieve elements and 93% of the total time with the stylet inserted into plant tissue. Klingauf (1987a) suggest that aphids are more adapted to a sessile way of life than a mobile one. The results from the behavioural analysis of three clones of *M. persicae* agree with these observations.

The high reproduction rate of *M. persicae* increased the number of individuals in the filming arena and within 3 days there was not enough space for both nymphs and adults. Clone R3 responded to crowded conditions with increased mobility. The phenomenon was probably intensified by the set up of the arena, prohibiting escape. The phenomenon was reversible and disturbed aphids developed the typical feeding

behaviour once the offspring were removed. *M. persicae* has been reported to disperse in repose to tactile stimulation (Matsuka and Mittler, 1978).

Increased mobility was not observed for clone S. Clones of the pea aphid (*Acyrthosiphon pisum*) have been reported to differ in the sensitivity to crowding (Lowe and Taylor, 1964; Sutherland, 1969). However, in the case for the S clone, the crowding did not appear to be the issue due to a low reproductive rate.

The production of honeydew was studied for clones S and R3 and a clear inverse relation with the level of mobility was revealed for both clones. Klingauf (1987a) also noted that during changes of feeding sites the excretion was interrupted for a few hours.

The honeydew excretion rate of the two clones was also investigated. For both clones, in 70% of the cases the honeydew drops were excreted with a rate ranging between one drop every 20 to 50 minutes. For clone R3 the most frequent interval between honeydew drops was 25 min and for S clone 35 min.

Excretion rate depends on numerous factors like, the stage of the insect, the host plant and its physiological status, and environmental parameters (temperature, humidity and atmospheric pressure) (Klingauf, 1981; Klingauf, 1987a). Klingauf (1981) working with *Aphis fabae* studied the excretion rate of honeydew on whole plants and reported a greater variation in the excretion frequency (2- 25 in 12h) depending on the effect of these internal and external factors. His maximum reading (25), however, translates to 1 drop every 25-30min, which is agreement with the current findings. Most of the

factors affecting excretion rate where controlled during the present work to highlight any differences between the excretion rate of the two clones.

Aphids take advantage of the pressure of the phloem sap when feeding on whole plants. However, they can control their feeding rate (Klingauf, 1987b), and when the pressure is negative or ambient (artificial diets, excised leaf disks), aphids can pump fluids into their alimentary canal (Dixon, 1998). Studies on aphid feeding behaviour using EPG also show the same phases and patterns, for both experiments conducted on excised leaves or whole plants (Powell, 1993; Tjallingii, 1985; Tjallingii and Hogen Esch, 1993). Therefore, the studies in the present work, on the honeydew excretion rate and on the extent of feeding behaviour, done with aphids on excised leaf disks can be considered as an established and reliable method in aphids' behavioural studies.

The rate of honeydew excretion was a strictly conserved phenomenon in aphid activity patterns as was illustrated both by using observational and tracking methods. Mobility does affect the number of drops produced over a time period, but the frequency of drop excretion is not affected and it is immediately reinstalled once the insects starts feeding. Birth of offspring also did not affect the excretion rate of honeydew.

Aphids can achieve very high reproductive rates. Factors affecting the reproductive rate (e.g. adults size, food quality, temperature and morph) described by Dixon (1987) were controlled during the experiments. It was not possible to study the reproductive rate of S clones. There were statistically significant differences between the

reproductive rates of clones R1, R2, and R3 clones. However, there was no consistent relationship between levels of insecticide resistance and rate of reproduction. Clone R2, had a higher reproduction rate from both, more resistant (R3) and less resistant (R1) aphid clones.

Variation in reproductive activity between aphids clones have been associated with the numbers of ovarioles and the morph (Wellings *et al.*, 1980). The green and pink clones of the pea aphid were reported to differ in their reproductive activity, but not in a consistent way (Dixon, 1985). However, in the present work reproductive rate of *M. persicae* was not affected by insecticide resistance.

At this point it should be noted that the *M. persicae* clones used in the present work differ only in the number of copies of the gene encoding for the esterase. All insecticide resistant aphid clones studied have identical esterase genes (Field *et al.*, 1994; Field *et al.*, 1993) and absence of genetic variation between clones of *M. persicae* has been confirmed (Al-Aboodi and ffrench-Constant, 1995). Subsequently the studies on these particular clones can be representative of the majority of *M. persicae* clones world-wide.

In summary, the behaviour of *M. persicae* susceptible and resistant clones was studied under optimal growth conditions. There was no evidence of differences among the clones in any of the aspects of biology investigated. From the present work there is no evidence that overproduction of esterase disadvantages a resistant clone. However, it should be said that comparative studies on aphid biology under poor or stressed

growth conditions might reveal differences between the clones, important for survival in the field.

Evidence of reduced sensitivity of the nervous system of some resistant aphid clones to stimuli has recently been associated with a sodium-channel mutation (kdr). This kdr factor occurs in combination with high esterase production, indicating a strong insecticidal selection, favouring aphids with multiple mechanism of resistance (Devonshire *et al.*, 1998; Field *et al.*, 1997; Foster *et al.*, 1997; Foster *et al.*, 1999). This is the only fitness trade off, reported for insecticide resistant aphids, at this time.

4.4.2 Effects of fungal infection on aphids behaviour

Under low environmental RH, aphids inoculated with Vertalec showed similar mobility and honeydew production, to the control (untreated) aphids. Under these unfavourable conditions for fungal growth, the presence of the pathogen did not affect the insect's behaviour. However, the fungus was able to infect the insects, once humid conditions were again present.

The requirement for high humidities for germination of *V. lecanii* spores has been noted by Hall (1981). Milner and Lutton (1986) demonstrated also that *V. lecanii* depends on high humidity for host infection and cadaver sporulation on *M. persicae.* Drummond *et al.* (1987) found that even short dry periods (70% RH) after spore application can affect the pathogenicity of *V. lecanii* on white fly (*Trialeurodes vaporariorum*).

Under high humidity conditions the fungus (Vertalec) proceeded to infect aphids. Mycosed insects showed signs of increased mobility 2 days after spore application.

This response was recorded consistently in both visual observation and tracking experiments. In later stages of the infection aphid behaviour followed one of two patterns. One aphid group showed reduced honeydew production with time and mobility was absent for two days prior to death. The other group of aphids did not show this reduction and both excretion and mobility were maintained. Movement events in the late stages of the experiment were characterised as pre-death behaviour; this ended abruptly in death.

It looks as if two different response patterns can be identified between mycosed insects, caused by the same isolate. One is more intense, resulting in reduced mobility and feeding and earlier death. The other infection process, does not affect the insect's feeding, but probably develops slowly until there is a sudden loss of co-ordination and eventual death. These insects lived longer and if *in planta* they would continue to cause damage to the host.

It has been reported that reduction in feeding can be one of the first overt changes in an infected host (Hajek, 1989; Moore *et al.*, 1992). However, in other insect-fungus interactions loss of appetite appears to be a symptom of infection in its late stages (McCoy *et al.*, 1988; Tanada and Kaya, 1993). Indeed, the food consumption for *Pluttella xylostella* larvae was affected 3 days after infection with *Zoophthora radicans* (1 day prior to death) (Furlong *et al.*, 1997). However, in the present work, both early and late reduction of feeding rate of infected aphids has been observed.

The two response patterns described previously could possibly reflect the effects of two different infection patterns exhibited by *V. lecanii* (Vertalec). Preliminary

experiments in Chapter 3 indicated differences in the attack mode of *V. lecanii* depending on the nutrient levels of the aphid cuticle. On cuticle, rich in sugars , *V. lecanii* exhibits extensive surface saprophytic growth. Schreiter *et. al* (1994) suggest that the high enzyme activity of *V. lecanii* on the cuticle can provide sufficient nutrients to support and encourage hyphal growth, rather that host invasion. Extended surface growth can damage and eventually kill the host. However, in this case of infection, the host functions would be very little affected, until the complete degradation of the cuticle and the consequent death, as described above, in the second host response pattern. In absence of nutrients, *V. lecanii* have been observed to invade the host directly (Chapter 3). In that case, fungal growth in the insect's haemocel, can suppress nutrient availability, produce toxins (effects on the appetite) and infect tissues and muscles (effects on mobility or control of sap ingestion and excretion). Effects on mobility and on honeydew excretion have been described in the first response pattern of infected aphids.

Loss of co-ordination was observed in infected adult aphids a few hours prior to death. Such effects in late stages of fungal infection have been described by McCoy *et al.* (1988). These events may relate to tissue (muscles) degradation by the fungal pathogen and to neurotoxic effects from secondary fungal metabolites (toxins). Destruxins, toxins produced by *M. anisopliae* have been reported to have an immediate knockdown effect when injected to on their host (*Manduca sexta*) (Samuels *et al.*, 1988).

A significant reduction in the offspring production was observed on day 5 followed by complete halt on day 6. This accounted for a rapid reduction in fecundity 1 day

prior to death. Further data analysis of reproductive rates at the individual level, confirmed that aphids showed reduced ability to reproduce one day before death. Yokomi and Gottwald (1988) found that isolates of *V. lecanii* reduced fecundity of *M. persicae, Aphis gossypii* and *Aphis citricola.* They thought that this was an effect of the weakened condition of the moribund adults. Yokomi and Gottwald (1988), Hall (1976b) and this author observed adult aphids giving birth to healthy nymphs while partially covered in sporulating fungus.

Bye (1999) using immunostaining techniques localised fungal protease in sections of infected aphids. No enzyme activity was found close to the ovarioles or the embryos, independent of the stage of infection and level of degradation of other surrounding tissues. This suggests that infected aphids will be able to produce healthy progeny, up to 4 days after application. In field trials this is a considerable disadvantage of *V. lecanii* as a control agent of aphids.

Mycosed aphids showed increased mobility, 1 to 2 days after inoculation. Aphids treated with either Vertalec or KV42 covered significantly longer distance in that period compared to control insects. This is clearly not a pre-death behaviour, since mobile aphids can settle again and feed normally. Possibly the increased levels of activity in the restricted filming arena might reflect what would be elevation-seeking behaviour *in planta.*

Infected insects have been reported to alter their activity levels (Tanada and Kaya, 1993). Fungus infected aphids in particular have been observed to move to elevated position (Harper, 1958; Rockwood, 1950; Samson *et al.*, 1988). However, most of

these responses have been reported in late stages of the infection (Hajek and St Leger, 1994; McCoy *et al.*, 1988; Tanada and Kaya, 1993). *V. lecanii* has not yet been reported to cause any kind of alteration on the activity levels of infected aphids. In general it is not yet understood what is causing the behavioural alterations and if there is any selective pressure that favoured their evolution (Horton and Moore, 1993). Compounds produced by the fungus or by the insect as a defensive act (toxins, enzymes) are suspected to influence these behavioural effects (Hajek and St Leger, 1994).

Differences were observed in the duration and the intensity of the effects on the aphid's activity induced by the two *V. lecanii* isolates. Vertalec infected aphids showed increased activity for a longer period (days 2 and 3) compared to KV42 (day 2). Comparing the significance of the effects, KV42 caused a highly significant effect ($P<0.001$) compared to Vertalec ($P=0.008$ and 0.050). These differences could possibly be explained by the different attack strategies implemented by each isolate during the infection process (Charnley, *pers. com.*). It has been demonstrated that Vertalec (KV71) kills the host predominately by proliferation and degrading enzymes (chymotrypsin protease) are produced only when in need of nutrients or for the exit of the fungus through the cuticle. In contrast, the KV42 attack strategy relies mainly on enzymes. KV42 was the first to invade the host, however minimal development was observed in contrast to the Vertalec isolate. Enhanced enzyme activity and absence of tissue degradation suggests that the KV42 isolate used enzyme activity to provide nutrients and also used enzyme activity and secondary metabolites (toxins) to kill the host (Bye, 1999, Graystone, *unpubl.*). The short, early, and intense behavioural effects of KV42, can be related to its invasion strategy (early enzyme activity and host attack

using toxin production). In the same way, the attack strategy of Vertalec isolate (invasion and proliferation) can relate to a less intense but longer duration host response. However, aphids responded both to fungal growth and the presence of toxins in their haemolymph.

CHAPTER FIVE

Improving secondary spore pick up using behaviour manipulation methods

5.1 Introduction

It is possible to alter the behaviour of a pest to protect a crop from damage. This is not a new concept but lately there has been an increased interest in behavioural manipulation for pest management. The idea is to reduce the dependence on broad-spectrum insecticides that are hazardous to humans and their environment and develop other more benign, efficient and selective protective agents. On a practical level the development of insecticide resistance underlines the need to provide alternatives to pesticides (Pickett, 1991).

Foster and Harris (1997) define behavioural manipulation as the use of stimuli that either promote or inhibit a particular behaviour and thereby change pest status. The definition excludes induced changes in host physiology and affects on crop growth, which create asynchrony between host and pest.

According to Foster and Harris (1997) there are three principal elements of a behavioural manipulation method:

a) the behaviour of the pest,

b) the means by which the behaviour is manipulated appropriately and,

c) the procedures that utilises the behavioural manipulation for protection of a resource from the pest.

Choosing the behaviour

In theory any behaviour through out the life cycle of the insects might be altered. This can aim directly to protect the crop (e.g. feeding behaviour leading to reduced damage) or indirectly (e.g. mating behaviour leading to reduced pest numbers). The behaviour that is eventually chosen to be manipulated is directly related to the availability of the appropriate means to do so.

Choosing the stimulus

Behaviour may be manipulated by changing the inputs the sensory system of the insect receives from the surrounding environment. One might also change the processing of the inputs by the nervous system (internal stimuli), however, this is generally inaccessible at this time (Foster and Harris, 1997).

A successful stimulus must be attended by a number of attributes; the following are described by Foster and Harris (1997):

Accessibility: The insect is able to perceive the stimulus

Definability and reproducibility: Defined mode of action that can be reproduced artificially

Controllability: Ability to control parameters of the stimulus, especially intensity and duration

Specificity: On the insect and of the particular behaviour

Practicability: Compound stability, flammability of the formulation, environmental hazards and similar properties (not directly related to the impact on insect behaviour) could affect the method's potential use.

There are different methods to utilise manipulation of behavioural stimuli for crop protection. One of the most widely used methods is the 'attract and annihilate'. The strategy is simple and involves attracting the insects to a site and removing them from the environment. Most commonly used attractants are volatile chemicals (e.g. sex pheromones) or visual stimuli or a combination of the two. Auditory stimuli also have being used but it is not common because of the very limited range of pests that it can be treated and the cost of the sound-generating equipment. Removal of the insects from the environment involves trapping or killing them with toxic compounds or pathogens (Foster and Harris, 1997).

Another method used is disruption of behaviour using attractants or repellents. Exclusively chemical stimuli are used in this method. Attractants disrupt the searching behaviour of insects (for host or mate). Repellents also affect the searching behaviour and act in a more direct way. Repellent chemicals can be plant volatiles, artificial compounds (e.g. fungicides, insecticides) or insect volatiles (aphid alarm pheromone).

Some insecticides are designed to repel rather than killing the insect (e.g. antifeedants). Neurotoxic insecticides (poisons) also affect the insect's behaviour but their aim is to kill. However both result in reducing the pesticidal insect behaviour. Sublethal doses of neurotoxic insecticides also influence insect's behaviour (see review by Haynes, 1988).

Feeding itself is a result of behavioural components like host finding, landing and host acceptance. Therefore, it is to be expected that neurotoxins will decrease feeding in ways not yet established. Few cases have been reported where food intake was increased after exposure to sublethal doses of insecticides.

The reproductive behaviour is also affected by sublethal doses of neurotoxins. Essentially every class of insecticide has been shown to decrease production of viable offspring or reduce oviposition. This could be a result of effects on feeding, mate finding, spermatogenesis, sperm mortality, ovulation etc. (Haynes, 1988).

Neurotoxins can stimulate or depress an insect's locomotory behaviour, such as walking or flight. Hyperactivity and uncoordinated movement are typical symptoms of intoxication but insects can perceive insecticides through sensory processes. It is very difficult to distinguish between repellency (sensory channel affects) and irritancy (neurotoxic events). Usually terms like repellent, irritant, antifeedant and stimulant are used to describe responses even when it is not known which of the two effects is really involved (Haynes, 1988).

5.1.1 Behavioural manipulation of aphids

It is possible to prevent aphids from infesting crops, prevent aphid probing, or modify probing to time intervals short enough to prohibit virus transmission by using methods of behavioural manipulation (Gibson and Rice, 1988).

Virus transmission is one of the main damaging consequences of aphid infestations. Generally neither aphicides nor host plant resistance are effective at preventing spread of non-persistently transmitted plant viruses. Indeed some aphicides promote brief probing and pest dispersal. Behavioural manipulation methods could provide environmentally safe means of control combining aphid control and minimal virus spread (Gibson and Rice, 1988).

Visual stimuli

Airborne alatae are attracted to orange-yellow-green light reflected by leaves and it is possible to reduce aphid infestation up to 60% with yellow sticky traps. Although yellow surfaces dipped in fast-acting synthetic pyrethdoids have been used for aphids control (Cohen and Marco, 1973) untreated yellow traps are used mainly for monitoring pest population levels and pest control for indoor crops (glasshouses).

Aphids are also repelled by white or reflecting surfaces. Therefore covering a crop with white netting can reduce virus incidence by 70-90% (Loebenstein and Raccah, 1980).

Chemical stimuli

Aphids are attracted to plant odours. Component chemicals particularly from primary hosts can be used to bait trap or improve the efficiency and/or the selectivity of yellow water traps (Chapman *et al.*, 1981).

Plant chemicals can also repel aphids (see review by Pickett *et al.* 1992). Extracts from neem seeds (*Azadirachta indica*) halved the numbers of *M. persicae* on treated leaves (Griffiths *et al.*, 1978). Apart from antifeedant activity, azadirachtin affects both fecundity and ability of virus transmission (Lowery *et al.*, 1997; Lowery and Isman, 1996; Nisbet *et al.*, 1994; Nisbet *et al.*, 1993). Further *Azadirachta indica* metabolites interfere with the host-endosymbiont relationship and inhibit the transmission of potato leafroll virus by *Myzus persicae* (van den Heuvel *et al.*, 1998).

Polygodial, a natural constituent of *Polygonum hydropiper* L. is a repellent and can be detected by *M. persicae* without ingestion. Unfortunately the chemical lacks persistence (Rice *et al.*, 1983).

Carboxylic fatty acids with carbon chains lengths from C_8 to C_{13} are repellent to *M. persicae* particularly dodecanoic acid (C_{12}) (Briggs *et al.*, 1983; Sherwood *et al.*, 1981). Lower terpenes like the monoterpene geraniol and the sesquiterpenes farnesol and bisabolene inhibit settling, probing and offspring production (Gutiérrez *et al.*, 1997). Garlic and onion oils also inhibit settling of *M. persicae* (Hori and Harada, 1995). Methoxyphenols, like caffeic, sinapic acid and scopoletin, compounds of cereals also affect probing and ingestion of phloem sap in resistant cultivars by grain aphid *Sitobion avenae* (F) (Leszczynski *et al.*, 1995).

From a practical point of view, as a stand alone measure able to compete commercially with aphicides a repellent would have to act before aphids' probe. This would prohibit virus spread but would also need to be sufficiently persistent to provide protection thought out the aphid immigration period (Gibson and Rice, 1988). Such repellents could, complement already established method.

Aphid behaviour is also affected by insecticides. Changes in behaviour are important because they may reduce direct damage and virus spread though chemical treatments sometimes also enhance virus spread (see review by Gibson and Rice, 1988)

Pyrethroids have a knockdown effect and give very good control of virus spread. Deltamethrin increased aphid mobility within minutes but also reduced virus spread due to restlessness and paralysis. Organophosphorus and carbamate insecticides affected aphid behaviour without contact. Increased mobility was observed because of release of EBF induced by the presence of the insecticides. The above effects can be altered depending on the level of insecticide resistance of the aphids involved (Gibson and Rice, 1988).

5.1.2 Aphid alarm pheromone

Pheromones are perhaps the best known of behaviour-controlling chemicals, otherwise known as semiochemicals. They are usually blends of chemicals, that act as signals between members of the same species. When other species are also involved in the interaction the terms allomones, kairomones or synomones are used when the signal favours the producer, the receiver, or both, respectively (Pickett, 1991).

Aphid alarm pheromone is released from cornicle secretions when aphids are attacked by predators or parasitoids and respond with defensive or avoidance behaviour like dispersing or falling of the plant. The main chemical component of the pheromone of most aphid species is (*E*)-*β*-farnesene (EBF) (Figure 5.1). The sixth antennal segment is involved in reception of the chemical (Pickett *et al.*, 1992).

EBF is a ubiquitous plant chemical. The essential oil of the hop plant, *Humulus lupulus,* contains EBF, but its activity is inhibited by (−)-β-caryophyllene, another sesquiterpene hydrocarbon, related biosynthetically to EBF. The aphid *Phorodon humuli* (Shrank) is unaffected by these compounds since it is a major pest of the hop (Dawson *et al.*, 1984). *Solanum berthaultii*, a close relative of the potato *Solanum tuberosum* releases EBF from foliar type B trichomes and non-host aphids respond by displaying typical alarmed behaviour (Gibson and Pickett, 1983). Interestingly isothiocyanates were found to synergise the activity of EBF (Dawson *et al.*, 1986).

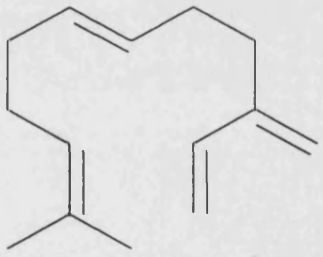


Figure 5.1 The aphid alarm pheromone, (E)-β-farnesene (Crock *et al.*, 1997).

EBF can be prepared from the commercially available nerolidol (Dawson *et al.*, 1982). Pickett *et al.* (1992) suggests that plants produce EBF from farnesyl pyrophosphate. Crock *et al.* (1997) isolated the cDNA clone of the sesquiterpene synthase for EBF from peppermint (*Mentha x poperita* L.), and expressed the gene in the bacterium *Escherichia coli*.

EBF is too labile to be of use in practical crop protection (Pickett *et al.*, 1992). However, semiochemicals like EBF could be integrated with other agents (chemical or biological) to produce highly efficient pest control (Pickett *et al.*, 1989). This practise has been established with EBF. Laboratory studies showed that increased aphid mobility improved pick up of pesticides (Griffiths and Pickett, 1980). Similar results were achieved in field experiments using a combination of an electrostatically applied hexane formulation of EBF with a synthetic pyrethroid. EBF also improved control of *Aphis gossypii* by the fungal pathogen *V. lecanii* (Griffiths and Pickett, 1987). It has been assumed that increased movement of aphids resulted in enhanced pick up of spores, but this remains to be confirmed.

5.1.3 Effects of imidacloprid on aphid behaviour

Imidacloprid (Gaucho®, Confidor®, Admire®) is a well-established insecticide being registered in more than 70 countries for foliar and soil application and seed treatment (Nauen and Elbert, 1994; Nauen *et al.*, 1998b). Imidacloprid is a member of a new chemical class of insecticides, the chloronicotinyl insecticides and the active ingredient is cyclic nitroguanidine, the 1-((6-chloro-3-pyridinyl)methyl)-N-nitro-2-imidazolidinimine (Figure 5.2) (Gardner and Kinard, 1998; Nauen, 1995). It acts by binding selectively to insect nicotinic acetylocholine receptors (nAChR) (Bai *et al.*, 1991; Liu and Casida, 1993; Liu *et al.*, 1993). It is particularly effective against homopteran pests such as aphids, leafhopers and whiteflies (Elbert *et al.*, 1996; Nauen *et al.*, 1996). Imidacloprid is also effective against other sucking insects like thrips, and chewing insects such as Coleoptera and microlepidopteran species (Elbert *et al.*, 1991; Nauen, 1995). It has no effect against nematodes or spider mites (Elbert *et al.*, 1991).

Imidacloprid and nicotine are believed to have the same mode of action, however imidacloprid has low mammalian toxicity compared to nicotine (Leicht, 1993). This unconventional mode of action gives excellent control of resistant homopteran, unaffected by the established resistance mechanisms (Nauen *et al.*, 1996).

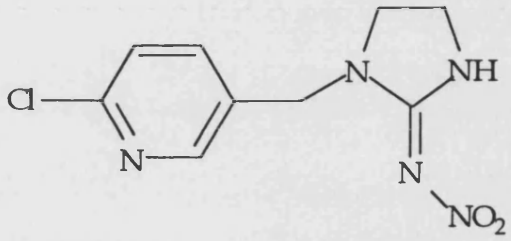


Figure 5.2 Structural formula of insecticide imidacloprid (Nauen *et al.*, 1998c)

At recommended field rates imidacloprid causes homopteran insects to die quickly through interference with nerve transmission while exhibiting typical irreversible symptoms of neurotoxicity (Bai *et al.*, 1991; Leicht, 1993; Liu and Casida, 1993; Liu *et al.*, 1993; Nauen, 1995). Sublethal doses of imidacloprid elicit behavioural changes in *M. persicae*. Aphids, feeding on plant tissue infused with sublethal doses of imidacloprid solution, showed decrease in weight, depression of honeydew excretion and restless behaviour. Ultimately aphid death from starvation occurred if the insects were left on the treated tissue. When the aphids were transferred to untreated leaves, they recovered well. The reversible starvation response was an antifeedant effect rather than symptoms of neuronal disorder (Nauen, 1995). Recently similar antifeedant effects as well as effects on reproduction at even lower concentrations were described for *Myzus nicotianae* (tobacco aphid) and *M. persicae* (Devine *et al.*, 1996; Nauen and Elbert, 1997). Time-lapse video filming of apterous adult aphids given a choice of systemically treated (low concentration of imidacloprid) and

untreated leaves revealed a migration to the untreated leaf (Nauen, 1995, Siskos and Reynolds, unpubl). Imidacloprid also affected cotton aphid, *Aphis gossypii* but it did not have any antifeedant effect on the Russian wheat aphids, *Diuraphis noxia* in field conditions (Burd *et al.*, 1996; Nauen and Elbert, 1994). Imidacloprid has been also reported to affect the feeding behaviour of the cotton boll weevil, *Anthonomus grandis* and the tobacco whitefly *Bemisia tabaci* (Nauen *et al.*, 1998b) and the false wireworms, *Somaticus* spp (Drinkwater, 1994).

Soil application of imidacloprid gave good and long lasting control of Colorado potato beetle, *Leptinotarsa decemlineata* (Say) and three potato colonising aphids (Boiteau *et al.*, 1997). The lasting residual activity of imidacloprid was justified by the presence of certain plant metabolites of imidacloprid with high oral activity against *M. persicae* and *Aphis gossypii* (Nauen *et al.*, 1998c).

Use of chemicals as 'stressors' to enhance the efficacy of mycopathogens has been proposed many times (e.g.Anderson *et al.*, 1989; Hassan and Charnley, 1989; Hassan *et al.*, 1989; Quintela and McCoy, 1998a). The synergism manifests itself in mortality levels elevated above those obtained following exposure to the insecticide or the fungus alone (Gardner and Kinard, 1998). Synergistic interactions between the insecticide imidacloprid and entomopathogenic fungi have been detected in several insects (Kaakeh *et al.*, 1997; Steinkraus and Tugwell, 1997). Studies indicated that the observed synergism was related to behavioural alterations induced by imidacloprid. Boucias *et al.* (1996) showed that the synergistic interaction between imidacloprid and the fungus *Beauveria bassiana* detected in termites was due to disturbed grooming behaviour and other social activities common in termites. Removal of

fungal conidia via grooming is a defence mechanism of termites and body paralysis caused by imidacloprid inhibited this behaviour thereby increasing the susceptibility of the termites to the fungus. Quintela and McCoy (1997b; 1998b) demonstrated synergistic effects of imidacloprid on both *Metarhizium anisopliae* and *Beauveria bassiana* treated larvae of root weevil *Diaprepes abbreviatus* (Coleoptera). Larval mobility was inhibited by imidacloprid and again loss of conidia avoidance behaviour was the cause of the synergy. Further studies by Quintela and McCoy (1998a) revealed an additional factor favouring synergism; enhanced conidia attachment on the cuticle of the root weevil was observed with imidacloprid treated larvae. Nevertheless, Gardner and Kinard (1998) found that imidacloprid had no effect on *in vitro* germination and growth of these two entomogenous fungi, supporting the theory that the observed synergism is resulting from the altered host behaviour and enhanced conidia attachment.

5.1.4 Aims

In the present work the effects of sublethal doses of the insecticide imidacloprid on *M. persicae* were investigated. The aim of the work in this chapter was to study the effects of EBF and imidacloprid on the pathogenicity of *V. lecanii* on *M. persicae*. It was anticipated that increased movement brought about by these chemicals would result in greater pick up of spores from leaf surfaces (secondary pick up).

5.2 Materials and methods

5.2.1 Materials

The alarm pheromone, (*E*)-β-farnesene, (61% pure in hexane) was provided in glass ampoules by Rothamstead Experimental station. Technical imidacloprid (Zeneca Agrochemicals) was kindly provided by Prof. S. E. Reynolds (University of Bath). All experiments were carried out using the *M. persicae* clone R1 and *V. lecanii* isolate Vertalec. This aphid clone did not have the kdr target site insecticide resistance (Devonshire, *pers. com.*) that was shown to reduce the sensitivity of the insect to EBF (Foster *et al.*, 1999).

5.2.2 Methods

5.2.2.1 Effect of alarm pheromone on secondary pick up

A repli dish was set up with segments of pepper leaf embedded in water agar in 5 of the 1cm^2 compartments. The dish was sprayed with dH_2O (control), 10^6, 10^7 and 10^8 *V. lecanii* conidia/ml, then allowed to dry for 15 min (see Secondary pick up section in Chapter 2)

Approximately 5 aphids were placed in each chamber and left to settle. 1µl of EBF (in hexane) was introduced via a microsyringe onto small pieces of filter paper (diameter 2mm) into the centre of the sprayed compartment. Preliminary experiments showed that hexane can damage the leaf epidermal cells and possibly the fungal spores. 1µl of hexane was used as a control. The compartments were immediately sealed with Parafilm M® and two holes made above each compartment for ventilation.

All repli dishes were incubated at 23±1°C with photoperiod 18H L: 6H D. After 24h the aphids were transferred to clean individual chambers as described in Chapter 2. The mortality was estimated after a further 6-day incubation.

5.2.2.2 Effect of imidacloprid on aphid behaviour

The tracking set up that was developed to study the behaviour of aphids was used to investigate the effects of sublethal systemic doses of imidacloprid. The aphids were filmed for 12h from the time that they were introduced to the leaf surface of the filming arena. Imidacloprid was systemically applied to pepper leaves using the following method.

Imidacloprid stock solutions were prepared in acetone (0.5ml acetone/1mg of imidacloprid) and were subsequently diluted to the appropriate concentration with distilled water. The final dilution, used to infuse the leaves, was made up with tap water. Pepper leaves were cut and their petioles immediately immersed in 0.1ppm of imidacloprid (sublethal dose determined by Siskos and Reynolds, *unpubl.*) or water for the control treatment. After allowing 24h for infusion, leaf disks (5cm diameter) were cut and filming arenas were prepared (see 'Tracking set up' in Chapter 2). 5 adult aphids were placed in the arena, which was then placed under the camera. Time-lapse filming started once the image was centred and focused (mode 12; 15h of real time in 3h of videotape).

Data analysis was restricted to the distance covered by the aphids in the 12-hour period. Distance covered was considered as an indicator of the effects of sublethal systemic application of imidacloprid.

5.2.2.3 Effects of imidacloprid on secondary pick up

Disks were cut of leaves infused in 0.1ppm imidacloprid water solution ('Imidacloprid' treatment) or water ('Water' treatment) for 24h and placed on water agar in a 5 cm Petri dish. (see section Secondary pick up, Chapter 2).

12 Petri dishes were used for each treatment (6 experimental, 6 control). 8 to 10 aphids were applied onto each dish and were left to feed on the leaf surface for 24h. After that time interval the aphids were transferred to individual uninfected chambers in repli dishes, Mortality was determined after a 7-day incubation (23±1°C, 18h L: 6h D).

5.2.2.4 Investigating the case of synergism between *V. lecanii* and imidacloprid

This is a modified version of the direct impact application method (see Chapter 2). 5 cm Petri dishes were prepared as described in the previous experiment using 0.1ppm imidacloprid or water treated leaf disks. 12 dishes were prepared for each treatment (6 experimental, 6 control). 8-10 aphids were transferred to each dish and were left to feed on the leaf disks for 24h, as in the previous experiment. After the 24-hour period the aphids were sprayed directly with 1.5 ml of 2.1×10^6sp/ml conidia suspension or dH_2O (control treatment). The aphids were transferred to uninfected repli dishes, after allowing the sprayed suspension to evaporate (10 min). Mortality was assessed after 7 days of incubation (23±1°C, 18h L: 6h D)

5.3 Results

5.3.1 Effect of alarm pheromone on secondary pick up

Aphids were placed for 24h on the leaf surface sprayed with conidia. After the first 2 - 3h (setting period) the aphids were exposed to EBF, hexane or left untreated. Subsequently insects were transferred to a non-contaminated environment to allow mycosis to develop.

Mortality among aphids exposed to EBF was significantly greater than either hexane or untreated controls, at both spore concentrations (see Figure 5.3 and Table 5.1).

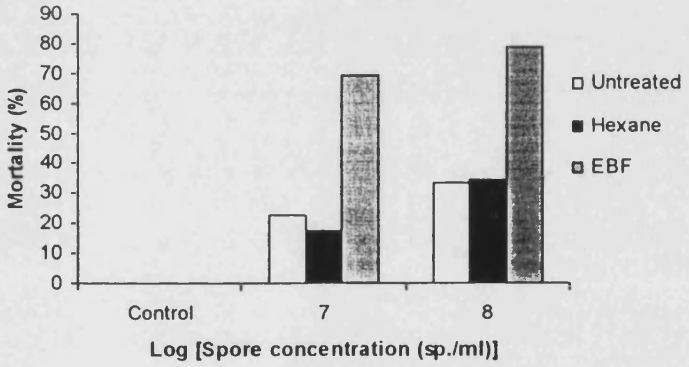


Figure 5.3 Aphid mortality by secondary spore pick up of *V. lecanii* for treatments Water, Hexane and EBF.

The graph illustrates the combined mortality recorded from 3 experiments (25 aphids per treatment). 'Hexane' = aphids exposed to solvent (hexane) alone, , and 'EBF' = aphids were exposed to the alarm pheromone in hexane.

Table 5.1 Statistical analysis of the effect of EBF on the aphid mortality in 'secondary pick up' experiments (see Figure 5.3).

Treatment		Spore concentration (sp./ml)					
		Control		10^7		10^8	
Untreated	Total dead	0	a	17	a	25	a
	Total alive	25		58		50	
Hexane	Total dead	0	a	13	a	26	a
	Total alive	25		62		49	
EBF	Total dead	0	a	52	b	59	b
	Total alive	25		23		16	

X^2 test applied for comparison between treatments Untreated, Hexane and EBF. Treatments within a column followed by the same letter are not significantly different ($P<0.05$).

The experiment was repeated six months later using the same batch of EBF. This time there were no significant differences between treatments suggesting lability of the pheromone (data not shown).

5.3.2 Effects of imidacloprid on aphid behaviour

Filming arenas were made with leaves infused in 0.1ppm imidacloprid water solution or plain tap water. 5 adult aphids were placed on each arena and filmed for 12h in time lapse mode.

Figure 5.4 shows the path travelled by individual aphids as revealed by the computer analysis of the captured images from the video recording. All three aphids showed searching behaviour in the first 4-h section (0-4h). In the second section (4-8h) only one aphid (N°2) kept on looking for a feeding place. The other two (N°1 and 3) had settle and were immobile for that 4h period (single dot on the respective graph). In the

last section (8-12h) all three had settled. This behaviour is typical of the aphid *M. persicae* introduced to a new feeding site.

Figure 5.5 shows a very different pattern of behaviour of aphids on imidacloprid treated leaves. Insects did not settle to feed. On the contrary there was a continuous and intense searching behaviour throughout the 12h filming period. Attempts by the aphids to settle failed (aphid N°2 in section (0-4h) and aphid N°1 in section (4-8h)).

The distance covered by the aphids was calculated for each of the three 4-h long sections (Figure 5.6). The behaviour of 20-22 aphids was analysed for both treatments. The data were not normally distributed, hence the median is used to describe the data.

The aphids on control (water) treated leaves showed mobility only in the first section of the 12h period. The median distance covered was 344 mm in the first 4h and 0 mm in both the following 4h periods (4-8 and 8-12h). In comparison aphids feeding on imidacloprid treated leaves were mobile through out the 12h filming period. The distance covered ranged between 280 to 460 mm over the 4h. Significant differences were detected between treatments for the later filming periods, 4-8 and 8-12h (Table 5.2).

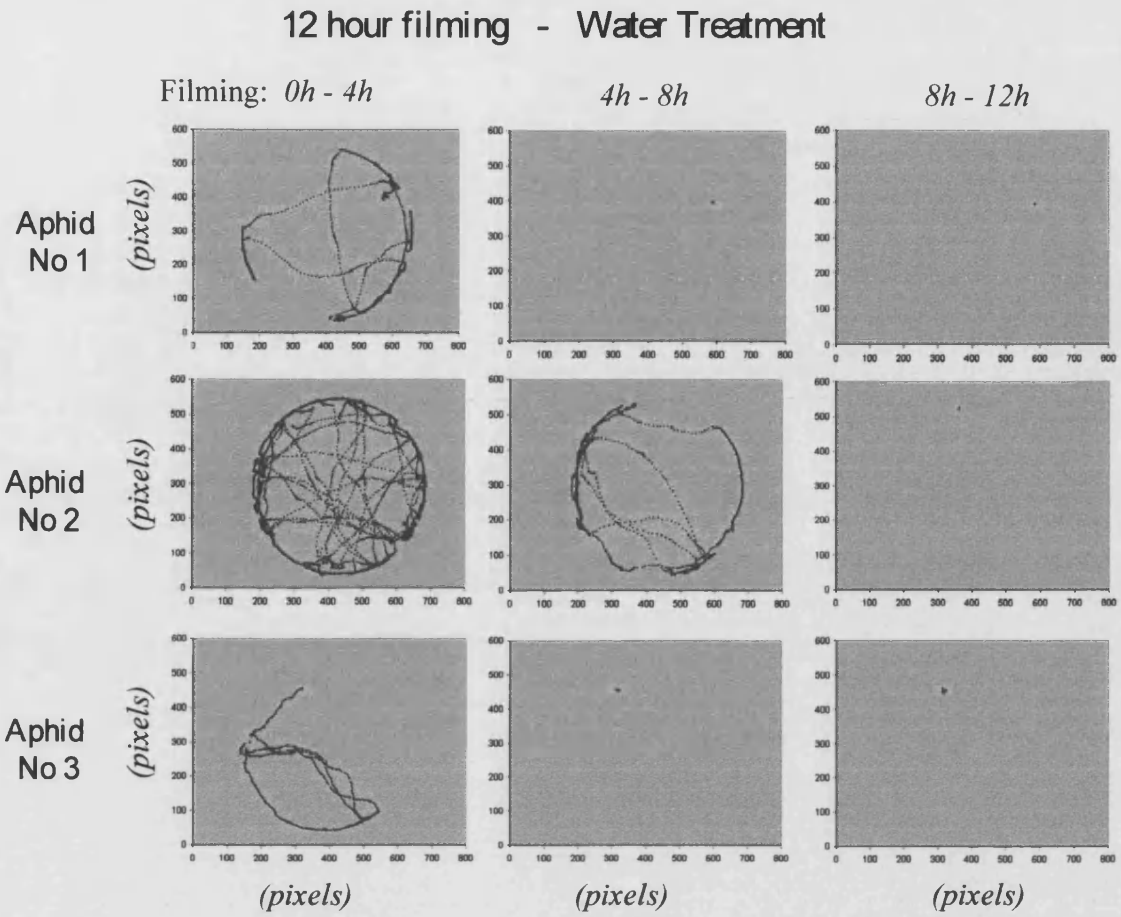


Figure 5.4 The tracks of the paths followed by three randomly selected aphids feeding on water treated (control) leaves in a 12 hour filming period.

The position of the aphid on the round area was recorded every second, by the tracking program, as *(x, y)* co-ordinates of the captured image. The co-ordinates are plotted as dots on the graphs, and the path followed by the aphid is revealed. The total distance covered can be calculated. On each graph, the path followed by a single aphid in a 4 hour filming period is displayed. The analysis of the total 12 hour of filming was done in 3 sequences of 4 hour long each. When a single dot is observed, the aphid did not move from the feeding position. Note that all three aphids were **immobile** in the third (8 - 12 h) section.

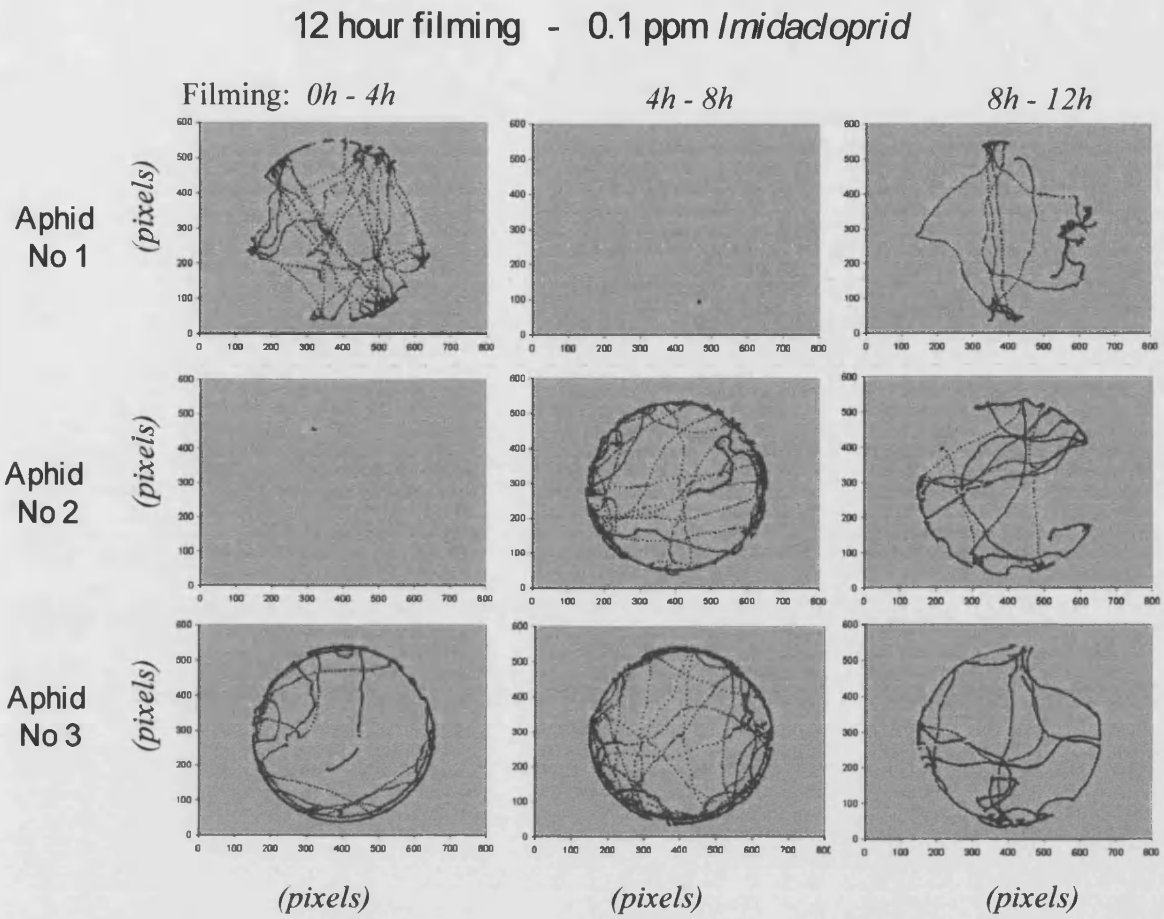


Figure 5.5 The tracks of the paths followed by three randomly selected aphids feeding on imidacloprid treated leaves in a 12 hour filming period.

The position of the aphid on the round area was, recorded every second by the tracking program, as *(x, y)* co-ordinates of the captured image. The co-ordinates are plotted as dots on the graphs, and the path followed by the aphid is revealed. The total distance covered can be calculated. On each graph, the path followed by a single aphid in a 4 hour filming period, is displayed. The analysis of the total 12 hour of filming was done in 3 sequences of 4 hour long each. When a single dot is observed, the aphid did not move from the feeding position. Note that all three aphids were **mobile** in the third (8 - 12 h) section.

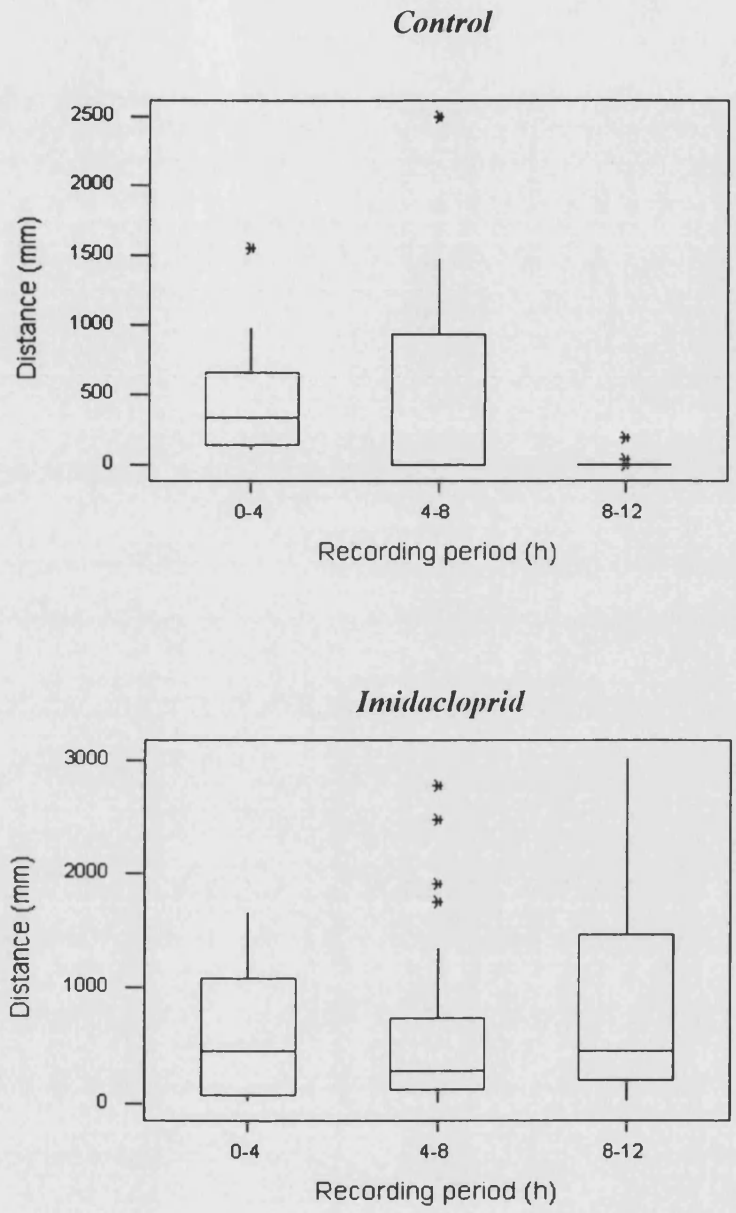


Figure 5.6 Distance covered by aphids in feeding on control (water) or imidacloprid treated leaves in the three sections of the 12h recording.

The median of the distance covered from about 20 aphids (data from all 5 replicates) for the two treatments are displayed in this graph. The 12h filming period was divided in three 4h long sections. The data were not normally distributed and for this reason the boxes represent the data median (line in the middle of the box) and the inter-quartile region (between the upper and lower box lines).

Table 5.2 Statistical analysis of the distance covered by aphids in feeding on control (water) or imidacloprid treated leaves in the three sections of the 12h recording

	Recording period		
	0 - 4	*4 - 8*	8 - 12
Control	344.3 *a*	0.0 *a*	0.0 *a*
Imidacoprid	459.6 *a*	277.1 *b*	455.7 *b*

The median distance covered in (mm) is displayed. The Mann-Whitney *U*-test for non-parametric data was applied. Data within a column followed by the same later are not significantly different ($P<0.05$).

5.3.3 Effects of imidacloprid on secondary pick up

This experiment was designed to study the impact of behavioural disturbance of aphids on imidacloprid treated leaves on the secondary pick up of spores from the leaf surface. Dishes with water or imidacloprid infused leaves were sprayed with $2.6x10^6$sp/ml or dH_2O then left to dry for 15 min before the aphids were applied (see section Secondary pick up, Chapter 2). Aphids were exposed to the conidia infected surface for 24h.

The mortality recorded for the treatments after the 7-day incubation period is displayed in Figure 5.7. Insects exposed to imidacloprid treated leaves infected with *V. lecanii* showed significantly greater mortality than insects on water treated leaves.

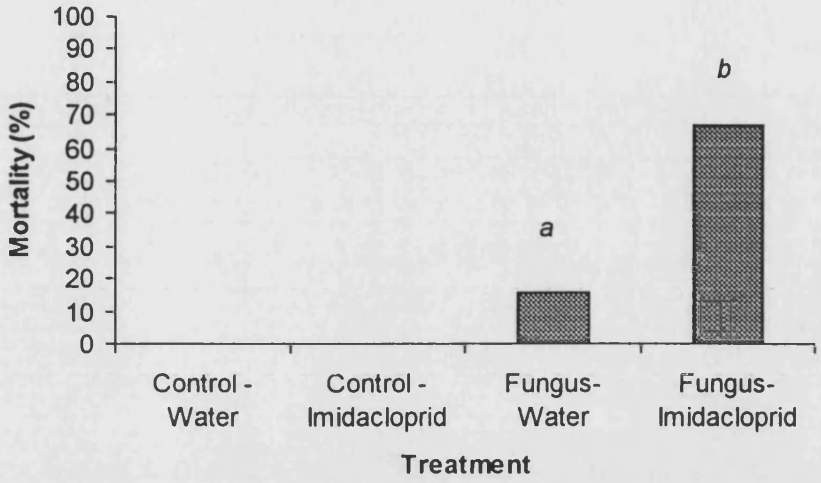


Figure 5.7 Aphid mortality by secondary pick up of *V. lecanii* conidia from leaf surface infused in water or 0.1ppm imidacloprid solution.

In this 'secondary pick up' experiments aphids (n=50 per treatment) were left to feed for 24h on *V. lecanii* infected leaf surfaces, treated either with water or imidacloprid solution. The *V. lecanii* dose applied was 2.5×10^6sp/ml (dH_2O for control treatment). The (%) mortality recorded after a further 6-day incubation is displayed on the graph. X^2 test was applied to the data. Treatments with the same letter are not significantly different ($P<0.05$).

5.3.4 Investigating the cause of synergism between *V. lecanii* and imidacloprid

The last experiment was designed to allow inoculum acquisition only by secondary pick up. However increased mortality among aphids on Imidacloprid treated leaves could be due to the detrimental effects of the insecticide rather than enhanced pick up of spores. Possible synergism between fungus and insecticide was investigated.

In this case it was important to have the same amount of fungal inoculum on the aphids independent of the presence of imidacloprid treated leaves; in contrast to the previous experiment. After the aphids were left to feed for 24h on water or

imidacloprid treated leaves, they were sprayed *in situ* with 10^6sp/ml *V. lecanii* spore suspension. The mortality after a 7-day incubation is illustrated in Figure 5.8. There was no control mortality and the difference between treatments was not significant.

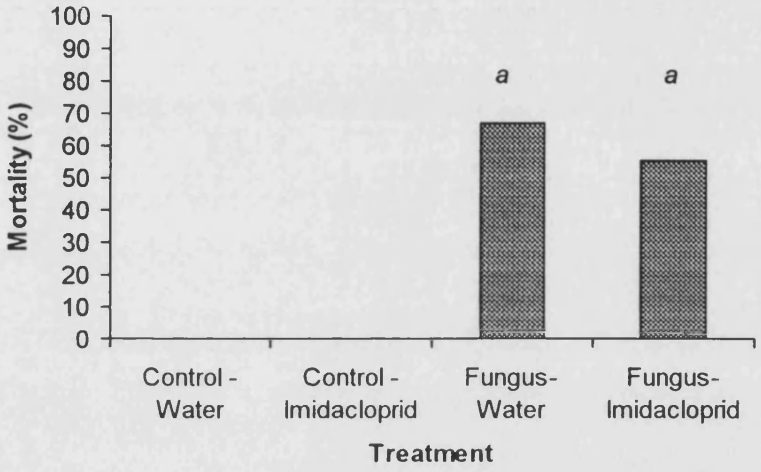


Figure 5.8 Aphid mortality recorded in direct impact experiment, investigating possible synergism between imidacloprid and *V. lecanii*.

The aphids (n=50 per treatment) were left to feed on water or imidacloprid infused leaves for 24h. Then the aphids were sprayed with 2.1×10^6sp/ml *V. lecanii* spore suspension (sterile dH_2O for control treatment) using direct impact application method. The graph illustrates the (%) mortality recorded after a further 6-day incubation. X^2 test was applied to the data. Treatments with the same later are not significantly different ($P<0.05$).

5.4 Discussion

Exposure to EBF significantly increased mortality among aphids in a situation where acquisition of a lethal dose of conidia could only occur from the leaf surface. This is the first critical laboratory experimental evidence for enhanced secondary pick up in response to EBF. The results support the field trials of Griffiths and Pickett (1980; 1987) who found enhanced aphid mortality when EBF was combined with pesticide or *V. lecanii* applications.

Reduced effectiveness of the pheromone experiments conducted 6 months later is consistent with the unstable nature of EBF (Pickett *et al.*, 1992). Indeed aphids did not demonstrate the typical alarm behaviour immediately after the EBF application in the second experiment as they did in the first.

It is almost three decades now since EBF was first discovered yet no use has been found for it in insect management. The reasons for this are complex but include the following. The effectiveness of the pheromone can be altered by the presence of plant compounds, acting as synergists (Dawson *et al.*, 1986) or as inhibitors (Dawson *et al.*, 1984). The response of aphids to the presence of the pheromone varies depending on the aphid species (aggregation density of the species, and whether the aphid is myrmecophilous) (Montgomery and Nault, 1977) or even on the clone of the aphid species (insecticide resistant clones show decreased response) (Dawson *et al.*, 1983).

Other reasons for the pheromone's failure to find practical use are difficulties in handling, storing and applying EBF. (*E*)-□-farnesene is a volatile, unstable and

flammable compound, which must be released continuously and in adequate volume in the field in order to obtain the desirable effect. However, even under the optimal application conditions aphids may accommodate to the presence of the pheromone and remain to colonise the crop (Gibson and Rice, 1988; Wohlers, 1981). Thus other methods need to be sought to increase mobility of aphids and to increase their chances of acquiring a lethal infection of a mycoinsecticde.

Analysis of the behaviour of aphids feeding on pepper leaves revealed a significant effect of the sublethal systemic dose of imidacloprid. Aphids on treated leaves demonstrated a continuous mobility through out the 12h long recording compared to aphids on control (water) treated leaves that settled in the first 4h. For the last 8h of the experiment, aphids on treated leaves were covering significantly more distance than aphids on water treated leaves. The presence of imidacloprid inhibited settling and subsequent feeding; this forced the insects into searching behaviour for an acceptable energy resource. Similar symptoms of restlessness and irritability were recorded by Nauen (1995), and Nauen *et al.* (1998c). It has been demonstrated that imidacloprid and its metabolites in the plant tissue have high oral antifeedant activity against *M. persicae* (Nauen, 1995; Nauen *et al.*, 1998c). Insects could starve to death on treated leaves. Given an option aphids chose the untreated leaves surface (Nauen, 1995, Siskos and Reynolds, *unpubl.*).

Significantly increased mortality occurred among aphids allowed to pick up spores from imidacloprid treated leaves compared to water treated leaves. High mobility of the insects on the treated surface appears to account for the increased aphid mortality, since no synergy was found between topically applied spores and systemic sublethal

imidacloprid. Furlong and Pell (1996) observed increased levels of infection of the Diamondback moth larvae, *Plutella xylostella* L. by the entomopathogenic fungus *Zoophthora radicans* Brefeld in the presence of the parasitoid *Diadegma semiclausum*. They suggested that the greater movement (as in distance covered and new areas visited) of the disturbed larvae accounted for increased inoculum pick up.

Imidacloprid has however been reported to synergise with entomopathogenic fungi against other insects. In a few cases the basis of the synergism has been examined (Boucias *et al.*, 1996; Quintela and McCoy, 1997a; Quintela and McCoy, 1997b; Quintela and McCoy, 1998b) though enhanced secondary pick up has not been demonstrated before.

Cost and compound stability are major factors in the choice of a compound to increase aphid movement. Imidacloprid has been on the market already for a number of years. Systemic applications of imidacloprid are accomplished easily by watering plants with insecticide solution. Imidacloprid has lasting effects since its metabolites themselves have high oral antifeedant activity against aphids (Nauen *et al.*, 1998c). The results, from the laboratory experiments, described here suggest that systemic applications of the insecticide with concentration of just 1% of the recommended dose could possibly improve the efficiency of *V. lecanii* application in field trials. Additionally, the combined use of control agents with different modes of action reduces the likelihood of simultaneous development of resistance to the two agents (Georghiou, 1994).

CHAPTER SIX

General discussion

The development of insecticide resistance is one of the major problems in pest control the last three decades (Lemon, 1994; Metcalf, 1989). However development of resistance, especially in the form of biochemical defence, as in the case of the overproduced esterase in resistant *M. persicae*, is possibly accompanied by a selective cost in the absence of the toxic agent (Bowers, 1992; Crow, 1957). In this study an alternative agent was used to control *M. persicae*, the fungal pathogen *V. lecanii*. The possible cost of insecticide resistance was investigated as expressed in the form of susceptibility differences to the fungus between aphid clones of different insecticide resistance levels.

The susceptibility of *M. persicae* clones to the fungal pathogen was tested by estimating mortality, using a repli dish bioassay. The bioassay results were affected by two independent factors related to the position of the aphids in the repli dish, the spore deposition (*in situ* experiments only) and the humidity conditions (RH) in each repli dish cell. Analysis of the results by zones indicated that the effects of these factors were consistent. Constant variability factors between treatments allowed comparisons between the aphid clones.

Three bioassay methods were followed. An *in situ* method aimed to give a general picture of any susceptibility differences between clones. All four aphid clones were tested, ultimately to test for a correlation between the levels of esterase production and susceptibility to the fungal pathogen. The results indicated no significant differences between the aphid clones and also no positive correlation, in the esterase/susceptibility relationship.

The direct impact bioassay method aimed exclusively at the susceptibility differences between the clones. The two most different resistant clones were tested, R1 and R3. The method was designed so that the aphids from both clones would be treated with same amount of inoculum. Increased variability was observed in the results, which was not related to the independent factors discussed previously. However, looking at the results critically, it was not evident that the two clones responded differently to the fungus. This was also supported by probit analysis of the overall results.

The secondary pick up bioassay method combined susceptibility differences with behavioural factors. Secondary pick up was thought to be related to the mobility of the insect (Hall, 1981; Milner and Lutton, 1986), and indeed this was demonstrated in Chapter 5. However, no susceptibility differences were observed between the clones when this bioassay method was applied. Combining this result, with the absence of susceptibility differences, demonstrated in previous bioassays, the author suggests that there are no differences in susceptibility to the fungus between the clones under the experimental (optimum) conditions.

Differences between susceptible and resistant aphid clones were studied further, on a behavioural level. The studies were focused on three levels; mobility, feeding, and reproduction rate. Mobility of both clones studied (S and R3) was remarkably low (1 movement event/aphid/day). Aphids were observed to feed continuously on the leaf tissue. Increased mobility was observed only under crowded conditions. When the tactile stimulation was removed, aphids exhibited typical feeding behaviour.

Excretion of honeydew was used as a measure of aphid feeding rate. Excretion events occurred in set time intervals; 70% of the intervals ranged between 20 - 50 min for both clones. Honeydew excretion (feeding) and mobility were inversely related.

Differences in offspring birth rate were observed between the aphid clones. However, there was no correlation with the levels of the esterase in the haemolymph conferring insecticide resistance. The observed differences in fecundity must be attributed to other factors.

In summary, overproduction of esterase was not shown in any case to disadvantage the insecticide resistant clones under optimal conditions. Kdr is the only resistance mechanism conferring selection cost to resistant *M. persicae*. However, this target-site mechanism affects the clone fitness indirectly, by reducing the insect sensitivity to various stimulus (e.g. tissue degradation and alarm pheromone) (Foster *et al.*, 1997; Foster *et al.*, 1999). Nevertheless, both resistance mechanisms, esterase detoxification and kdr, are closely related (Field *et al.*, 1997), suggesting that under field conditions resistant aphid clones will suffer the consequences of this fitness trade off.

V. lecanii did not affect the aphid offspring production or the honeydew excretion rate in the early stages of the development of the infection. Both functions showed a decline only 4 days after inoculation, 1-2 days prior to death. These results suggest that infected aphids are capable both of reproducing and damaging the crop, long after the inoculation application. This study highlights the major disadvantage of fungal pathogens, the slow kill time. The extent of this problem would be even greater in the field because of fluctuating environmental conditions affecting fungal growth (e.g. temperature humidity), which would not always be optimal, as in the present work.

The particular host-pathogen combination, failed to affect dramatically the feeding ability of the host, which was achieved in other cases (Moore *et al.*, 1992). However, a moderate reduction in feeding and mobility was observed in some cases. The inconsistency of the observations probably reflects the opportunistic nature of Vertalec isolate. Microscope observation of germinating spores showed that the fungus could choose between surface growth under optimal nutrient conditions, or host invasion in the absence of nutrients. Bye (1999), demonstrated that the presence of a carbon source inhibits the production of protease by Vertalec isolate. The protease is associated with host-cuticle degradation and penetration. Vertalec will attack the host by extensive growth either externally or internally, and that could result in differences in the host response to the infection.

In contrast to Vertalec, protease production in KV42 isolate was not inhibited by the presence of nutrients and was found to invade the host earlier, compared to Vertalec (Bye, 1999). KV42 showed restricted growth once in the host and high enzyme production. This suggested that this isolate is using protease activity not only in the

early stages, to invade the host, but also throughout the infection to provide nutrients and possibly to kill the host along with other secondary fungal metabolites. In contrast to Vertalec's attack strategy by proliferation, KV42 uses another strategy based on enzyme production.

These different isolate strategies had an impact on the host response to the infection. Both infection processes had a significant effect on the mobility exhibited by the host during the first days of infection. The response to the KV42 infection was short (1 day) but intense. The effect of Vertalec was longer (2 days) and comparatively less intense, but statistically significant.

These responses were observed under optimal conditions for fungal growth. It is possible that such responses will not be exhibited under less favourable growth environments in the field. Infection experiments under low RH did not indicate effects on the aphid behaviour. Also it is not known what exactly is the result of this increased mobility *in planta*. In response to fungal infection aphids have been reported to seek elevated position (Rockwood, 1950; Samson *et al.*, 1988). However, this behaviour has never been reported for *M. persicae* infection with *V. lecanii*. Possibly this mobility results in increased dispersal of the infected hosts. This could have a positive effect on the spread of the disease. Horizontal transmission was shown to be an effective form of infection spread between infected and uninfected aphid individuals. Nevertheless, if these mobile, infected, hosts act effectively to remove themselves from the colony and avoid typical aphid habitats, this could result in an adverse effect on successful establishment of the disease.

V. lecanii spore adhesion to the insect cuticle is a passive event and mainly depends on contagion (Boucias and Pendland, 1991; Hall and Papierok, 1982). Consequently, successful host infection also very much depends on accidental spore pick up. *M. persicae* is considered a relatively mobile, compared to other aphid species. Differences in the ability of *V. lecanii* to control aphid species in field trials were related to differences in their activity levels and subsequent accidental spore pick up (Hall, 1981; Milner and Lutton, 1986; Sopp *et al.*, 1990). However, behavioural studies illustrated that, *M. persicae* is also very much adapted to a sessile way of life. Using a method to increase such low aphid mobility could result in an increased efficacy of *V. lecanii* spore application.

The aphid alarm pheromone ((*E*)-β-farnesene) affects aphid mobility (Pickett *et al.*, 1989). Use of EBF in *V. lecanii* secondary pick up bioassays, increased aphid mortality, however, the results were inconsistent. These observations were related to the instability of the active compound (Pickett *et al.*, 1992). Recent work by Foster *et al.* (1999) indicated reduced response of resistant aphid clones to the alarm pheromone (as first observed by Dawson (1983)) was related to knockdown resistance (kdr). Insensitivity to stimulus of resistant clones is a disadvantage in the field (Foster *et al.*, 1997). However, if EBF was used as a way to increase mobility, this insensitivity would turn out to be in their favour. Additional problems related to production, storage, handling and application of EBF (Gibson and Rice, 1988; Wohlers, 1981), result in a complicated and unreliable method of behaviour manipulation.

Nauen (1995) showed that, sublethal systemic doses of the insecticide imidacloprid, had an antifeedant effect on *M. persicae*. This increased the mobility of the aphids resulted in a combined effect of inability to find a suitable feeding site and irritancy. Work in Bath University on imidacloprid produced similar results (Siskos and Reynolds *unpubl.*).

In the present work it was demonstrated that indeed, increased mobility, induced by sublethal doses imidacloprid, conferred increased mortality in secondary pick up experiments. Further studies excluded the case of synergism of *V. lecanii* with imidacloprid at the biochemical level suggesting that increased mortality resulted from increased accidental contagion with spores on the leaf surface.

Manipulation of insects' behaviour can be a complicated issue. In the case of *M. persicae*, behavioural studies indicated the strong tendency of this insect to remain immobile, which looked as a promising characteristic for manipulation. Increasing aphid mobility would seem to be quite straightforward: however, in practice it turned out to be fairly complicated, as in the case of EBF. Use of imidacloprid produced the desired results, by having a consistent and long lasting antifeedant effect on aphid behaviour. An additional advantage of the particular method is that it can be scaled up from a bioassay level, (using imidacloprid infused leaves), to field trials (using imidacloprid solution for watering the plants).

The efficacy of *V. lecanii* spore application was increased more than two-fold using behavioural manipulation. This could be increased further in combination with other improved methods (e.g. formulations, application) or improved isolates. Taking into

account that all aphid clones were found to be equally susceptible to the pathogen, *V. lecanii* could play a major role in aphid control in the future.

References

Abbot, W. S. (1925). A method of computing the efectivens of an insecticide. *Journal of Economic Entomology* **18**, 265-267.

Al-Aboodi, A., and ffrench-Constant, R. H. (1995). RAPD PCR confirms absence of genetic-variation between insecticide resistant variants of the green peach aphid, *Myzus persicae* (Homoptera, Aphididae). *Great Lakes Entomologist* **28**, 127-133.

Anderson, T. E., Hajek, A. E., Roberts, D. W., Preisler, H. K., and Robertson, J. L. (1989). Colorado potato beetle (Coleoptera, Chrysomelidae) - effects of combinations of *Beauveria bassiana* with insecticides. *Journal of Economic Entomology* **82**, 83-89.

Bai, D. L., Lummis, S. C. R., Leicht, W., Breer, H., and Sattelle, D. B. (1991). Actions of Imidacloprid and a related Nitromethylene on cholinergic receptors of an identified insect motor-neuron. *Pesticide Science* **33**, 197-204.

Balrow, C. A. (1962). The influence of temperature on the growth of experimental populations of *Myzus persicae* (Sulzer) and *Macrosiphum euphorbiae* (Thomas) (Aphididae). *Canadian Journal of Zoology* **40**, 145-156.

Bateman, R. P., Carey, M., Moore, D., and Prior, C. (1993). The enhanced infectivity of *Metarhizium flavoviride* in oil formulations to desert locust at low humidities. *Annals of Applied Biology* **122**, 145-152.

Bath, A. J. (1997). Temperature and infection by *Metarhizium* spp. in the desert locust., University of Bath, Bath.

Benzer, S. (1973). Genetic dissection of behavior. *Scientific American* **229**, 24-37.

Bidochka, M. J., St Leger, R. J., Stuart, A., and Gowanlock, K. (1999). Nuclear rDNA phylogeny in the fungal genus *Verticillium* and its relationship to insect and

plant virulence, extracellular proteases and carbohydrases. *Microbiology-Uk* **145**, 955-963.

Blackman, R. L. (1976). Biological approaches to the control of aphids. *Philosophical Transactions of the Royal Society of London Ser. B* **274**, 473-488.

Blackman, R. L. (1988). Rearing and handing Aphids. *In* "Aphids their biology, natural enemies and control." (A. K. Minks and P. Harrewijn, eds.), pp. 59-68. Elsevier, Amsterdam.

Blackman, R. L., Spence, J. M., Field, L. M., and Devonshire, A. L. (1995). Chromosomal location of the amplified esterase genes conferring resistance to insecticides in *Myzus persicae* (Homoptera, Aphididae). *Heredity* **75**, 297-302.

Blackman, R. L., Spence, J. M., Field, L. M., and Devonshire, A. L. (1999). Variation in the chromosomal distribution of amplified esterase (FE4) genes in Greek field populations of *Myzus persicae* (Sulzer). *Heredity* **82**, 180-186.

Blackman, R. L., Spence, J. M., Field, L. M., Javed, N., Devine, G. J., and Devonshire, A. L. (1996). Inheritance of the amplified esterase genes responsible for insecticide resistance in *Myzus persicae* (Homoptera: Aphididae). *Heredity* **77**, 154-167.

Blackman, R. L., Takada, H., and Kawakami, K. (1978). Chromosomal rearrangement involved in insecticide resistance of *Myzus persicae*. *Nature* **271**, 450-452.

Boiteau, G., Osborn, W. P. L., and Drew, M. E. (1997). Residual activity of imidacloprid controlling colorado potato beetle (Coleoptera: Chrysomelidae) and three species of potato colonising aphids (Homoptera: Aphidae). *Journal of Economic Entomology* **90**, 309-319.

Boucias, D. G., and Pendland, J. C. (1991). Attachment of mycopathogens to cuticle. *In* "The fungal spore disease initiation in plants and animals" (G. T. Cole and H. C. Hoch, eds.). Plenum Press, New York.

Boucias, D. G., Stokes, C., Storey, G., and Pendland, J. C. (1996). The effects of imidacloprid on the termite *Recticulitermes flavipes* and its interaction with the mycopathognes *Beauveria bassiana*. *Pflanzenshulz-Nachrichten Bayer.* **49**, 105-151.

Bowers, M. D. (1992). The evolution of unpalatability and the cost of chemical defense in insects. *In* "Insect Chemical Ecology: An evolutionary approach" (B. D. Roitberg and I. M. B., eds.). Chapman and Hall, New York, London.

Brey, P. T., Latgé, J. P., and Prevost, M. C. (1986). Integument penetration of the pea aphid *Acyrthosiphum pisum*, by *Conidiobolud obscurus* (Entomophthoracea). *Journal of Invertebrate Pathology* **48**, 34-41.

Briggs, G. G., Dawnson, G. W., Gibson, R. W., Griffiths, D. C., Pickett, J. A., Rice, A. D., Stribley, M. F., and Woodcock, C. M. (1983). Compounds derived from the aphid alarm pheromone of potential use in controlling colonisation and virus transmission by aphids. *In* "IUPAC Pesticide Chemistry" (L. Miyamoto and e. al., eds.), pp. 117-122. Pergamon Press.

Brobyn, P. J., Clark, S. J., and Wilding, N. (1988). The effect of fungus infection of *Metopolophium dirhodum* (Hom.: Aphididae) on the oviposition behavior of the aphid parasitoid *Aphidius phopalosiphi* (Hym.: Aphidiidae). *Entomophaga* **33**, 333-338.

Burd, J. D., Elliott, N. C., and Reed, D. K. (1996). Effects of the aphicides 'Gaucho' and CGA-215944 on feeding behaviour and tritrophic interactions of Russian wheat aphids. *Southwestern Entomologist* **21**, 145-152.

Butt, T. M., Carreck, N. L., Ibrahim, L., and Williams, I. H. (1998). Honey-bee-mediated infection of pollen beetle (*Meligethes aeneus* Fab.) by the insect-pathogenic fungus, *Metarhizium anisopliae*. *Biocontrol Science and Technology* **8**, 533-538.

Bye, N. J. (1999). Regulation of protease production in *Verticillium lecanii*, University of Bath, Bath.

Carver, M. (1988). Biological control of aphids. *In* "Aphids their biology, natural enemies and control" (A. K. Minks and P. Harrewijn, eds.), Vol. C, pp. 141-164. Elsevier, Amsterdam.

Chandler, D., Heale, J. B., and Gillespie, A. T. (1993a). Competitive interaction between strains of *Verticillium lecanii* on 2 insect hosts. *Annals of Applied Biology* **122**, 435-440.

Chandler, D., Heale, J. B., and Gillespie, A. T. (1993b). Germination of the entomopathogenic fungus *Verticillium lecanii* on scales of the glasshouse whitefly *Trialeurodes vaporariorum*. *Biocontrol Science and Technology* **3**, 161-164.

Chandler, D., Heale, J. B., and Gillespie, A. T. (1994). Effect of osmotic potential on the germination of conidia and colony growth of *Verticillium lecanii*. *Mycological Research* **98**, 384-388.

Chapman, R. F., Bernays, E. A., and Simpson, S. J. (1981). Attraction and repulsion of the aphid, *Cavariella aegopodii*, by plant odours. *Journal of Chemical Ecology* 7, 881-888.

Charnley, A. K. (1984). Physiological aspects of destructive pathogenesis in insects: a speculative review. *In* "Invertebrate - Microbial interactions. (British Mycological Society Symposium 6)" (J. M. Anderson, A. D. M. Rayner and D. W. H. Walton, eds.), pp. 229-270. Cambridge University Press.

Charnley, A. K. (1997). Entomopathogenic fungi and their role in pest control. *In* "The Mycota IV, Environmental and Microbial Relationships" (D. T. Wicklow and B. E. Södertröm, eds.), Vol. 4, pp. 185-201. Springer-Verlag, Berlin, Heidelberg.

Charnley, A. K., and St. Leger, R. J. (1991). The role of cuticle-degrading enzymes in fungal pathogenesis in insects. *In* "The fungal spore disease initiation in plants and animals" (G. T. Cole and H. C. Hoch, eds.). Plenum Press, New York.

Chon, J. H., Vizena, A. D., Rock, B. M., and Chaikof, E. L. (1997). Characterization of single-cell migration using a computer-aided fluorescence time-lapse videomicroscopy system. *Analytical Biochemistry* **252**, 246-254.

Clark, A. J. (1933). "The mode of action of drugs upon cells," Edward Arnold and Co, London.

Cohen, S., and Marco, S. (1973). Reducing the spread of aphid-transmitted viruses in peppers by trapping the aphids on sticky yellow polyethylene sheets. *Phytopathology* **63**, 1207-1209.

Crock, J., Wildung, M., and Croteau, R. (1997). Isolation and bacterial expression of a sesquiterpene synthase cDNA clone from peppermint (*Mentha* x *piperita*, L.)

that produces the aphid alarm pheromone (*E*)-β-farnesene. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 12833-12838.

Crow, J. F. (1957). Genetics of insects resistance to chemicals. *Annual Review of Entomology* **2**, 227-246.

Dadd, R. H., and Mittler, T. E. (1965). Studies on the artificial feeding of the aphid *Myzus persicae* (Sulzer). III. Some major nutritional requirements. *Journal of Insect Physiology* **11**, 717-743.

Davenport, D. C., Wright, C. A., and Causley, C. (1961). Technique for study of the behaviour of motile microorganisms. *Science* **135**, 1059-1060.

Dawson, G. W., Griffiths, D. C., and Pickett, J. A. (1984). Natural inhibition of the aphid alarm pheromone. *Entomologia experimentalis et applicata* **36**, 197-199.

Dawson, G. W., Griffiths, D. C., Pickett, J. A., J., W. L., and Woodcock, C. M. (1986). Plant compounds that synergise activity of the aphid alarm pheromone. *In* "Bridish crop protection conference - Pests and Diseases", Vol. 7C-3.

Dawson, G. W., Griffiths, D. C., Pickett, J. A., Smith, M. C., and Woodcock, C. M. (1982). Improved preparation of (*E*)-β–farnesene and its activity with economically important aphids. *Journal of Chemical Ecology* **8**, 1111-1117.

Dawson, G. W., Griffiths, D. C., Pickett, J. A., and Woodcock, C. M. (1983). Decreased response to alarm pheromones by insecticide resistant aphids. *Naturwissenschaften* **70**, 254.

Devine, G. J., Harling, Z. K., Scarr, A. W., and Devonshire, A. L. (1996). Lethal and sublethal effects of Imidacloprid on nicotine-tolerant *Myzus nicotianae* and *Myzus persicae*. *Pesticide Science* **48**, 57-62.

Devonshire, A. L. (1977). The properties of a carboxylestarese from the peach-potato aphid, Myzus persicae (Sulz.), and its role in conferring insecticide resistance. *The Biochemical Journal* **167**, 675-683.

Devonshire, A. L. (1989). Resistance of aphids to insecticides. *In* "Aphids their biology, natural enemies and control" (A. K. Minks and P. Harrewijn, eds.), Vol. C, pp. 123-139. Elsevier, Amsterdam.

Devonshire, A. L., and Field, L. M. (1995). Esterases in resistant peach-potato aphids, *Myzus persicae* (Sulzer). *Journal of Cellular Biochemistry*, 191 pp.

Devonshire, A. L., Field, L. M., Foster, S. P., Moores, G. D., Williamson, M. S., and Blackman, R. L. (1998). The evolution of insecticide resistance in the peach-potato aphid, *Myzus persicae*. *Philosophical Transactions of the Royal Society of London Series B - Biological Sciences* **353**, 1677-1684.

Devonshire, A. L., and Moores, G. D. (1982). A carboxylesterase with broad substrate-specificity causes organophosphorus, carbamate and pyrethroid resistance in peach-potato aphids (*Myzus persicae*). *Pesticide Biochemistry and Physiology* **18**, 235-246.

Devonshire, A. L., Moores, G. D., and Chiang, C. (1983). The biochemistry of insecticide resistance in the peach-potato aphid (*Myzus persicae*). *Pesticide Chemistry (Proc. 5th International Congress of Pesticide Chemistry)* **3**, 191-196.

Devonshire, A. L., Moores, G. D., and ffrenchc-Constant, R. H. (1986). Detection of insecticide resistance by immunological estimation of carboxylesterase activity in *Myzus persicae* (Sulzer) and cross reaction of the antiserum with *Phorodon humuli* (Schrank) (Hemiptera: Aphididae). *Bulletin of Entomological Research* **76**, 97-107.

Devonshire, A. L., and Rice, A. D. (1988). Aphid bioassay techniques. *In* "Aphids their biology, natural enemies and control" (A. K. Minks and P. Harrewijn, eds.), Vol. B, pp. 119-128. Elsevier, Amsterdam.

Devonshire, A. L., and Sawicki, R. M. (1979). Insecticide-resistant *Myzus persicae* as an example of evolution by gene duplication. *Nature* **280**, 140 - 141.

Dixon, A. F. G. (1985). Structure of aphids populations. *Annual Review of Entomology* **30**, 155-174.

Dixon, A. F. G. (1987). Parthenogenetic reproduction and the rate of increase in aphids. *In* "Aphids their biology, natural enemies and control" (A. K. Minks and P. Harrewijn, eds.), Vol. A, pp. 269-287. Elsevier, Amsterdam.

Dixon, A. F. G. (1998). "Aphid ecology," Second edition/Ed. Chapman and Hall, London.

Doberski, J. W. (1981). Comparative laboratory studies on three fungal pathogens of the elm bark beetle *Scolytus scolytus*: pathogenisity of *Beauveria bassiana*, *Metahrizium anisopliae* and *Paecilomyces farinosus*, to larvae and adults of *S. scolytus*. *Journal of Invertebrate Pathology* **37**, 188-194.

Domsch, K. H., Gams, W., and Anderson, T., eds. (1980). "Compendium of soil fungi.,". Acad. Press.

Drinkwater, T. W. (1994). Comparison of imidacloprid with carbamate insecticides, and the role of planting depth in the control of false wireworms, *Somaticus* sp., in maize. *Crop Protection* **13**, 341-345.

Drummond, J., and Heale, J. B. (1985). Vital staining of the entomopathogen *Verticillium lecanii* on a live insect host. *Transaction of the British Mycological Society* **85**, 171-173.

Drummond, J., Heale, J. B., and Gillespie, A. T. (1987). Germination and effect of reduced humidity on expression of pathogenicity in *Verticillium lecanii* against the glasshouse whitefly *Trialeurodes vaporariorum*. *Annals of Applied Biology* **111**, 193-201.

Du, Y. J., Poppy, G. M., Powell, W., and Wadhams, L. J. (1997). Chemically mediated associative learning in the host foraging behavior of the aphid parasitoid *Aphidius ervi* (Hymenoptera: Braconidae). *Journal of Insect Behavior* **10**, 509-522.

Elbert, A., Becker, B., Hartwing, J., and Erdelen, C. (1991). Imidacloprid - a new systemic insecticide. *Pflanzenschutz Nachrichten Bayer* **44**, 113-136.

Elbert, A., Nauen, R., Cahill, M., Devonshire, A. L., Scarr, A. W., Sone, S., and Steffens, R. (1996). Resistance management with chloronicotyl insecticides using imidacloprid as an example. *Pflanzenschutz Nachrichten Bayer* **49**, 5-55.

Esau, K., Namba, R., and Rasa, E. A. (1961). Studies on penetration of sugar beet leaves by stylets of *Myzus persicae*. *Hilgardia* **30**, 517-529.

Evans, H. C., and Samson, R. A. (1986). The genus Verticillium: Taxonomic problems in species with invertebrate hosts. *In* "Fundamental and applied aspects of Invertebrate pathology" (R. A. Samson, J. M. Vlak and D. Peters, eds.), pp. 186-189. Foundation of thr Fourth International Colloquium of Inveteberate pathology, Wageningen.

Feng, M. G., Poprawski, T. J., and Khachatourians, G. G. (1994). Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control - current status. *Biocontrol Science and Technology* **4**, 3-34.

Field, L. M., Anderson, A. P., Denholm, I., Foster, S. P., Harling, Z. K., Javed, N., Martinez-Torres, D., Moores, G. D., Williamson, M. S., and Devonshire, A. L. (1997). Use of biochemical and DNA diagnostics for characterising multiple mechanisms of insecticide resistance in the peach-potato aphid, *Myzus persicae* (Sulzer). *Pesticide Science* **51**, 283-289.

Field, L. M., Devonshire, A. L. (1997) Structure and organisation of amplicons containing the E4 esterase genes responsible for insecticide resistance in the aphid *Myzus persicae* (Sulzer). *Biochemical Journal* **322**, 867-871.

Field, L. M., Devonshire, A. L., and Forde, B. G. (1988). Molecular evidence that insecticide resistance in peach potato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene. *Biochemical Journal* **251**, 309-312.

Field, L. M., Devonshire, A. L., and Tyler-Smith, C. (1996). Analysis of amplicons containing the esterase genes responsible for insecticide resistance in the peach-potato aphid *Myzus persicae* (Sulzer). *Biochemical Journal* **313**, 543-547.

Field, L. M., Javed, N., Stribley, M. F., and Devonshire, A. L. (1994). The peach-potato *Myzus persicae* and the tobacco aphid *Myzus nicotianae* have the same esterase-based mechanisms of insecticide resistance. *Insect Molecular Biology* **3**, 143-148.

Field, L. M., Williamson, M. S., Moores, G. D., and Devonshire, A. L. (1993). Cloning and analysis of the esterase genes, conferring insecticide resistance in peach-potato aphid *Myzus persicae* (Sulzer). *Biochemical Journal* **294**, 569-574.

Finney, D. J. (1964). "Probit analysis," Cambridge University Press, Cambidge.

Foster, S. P., Denholm, I., Harling, Z. K., Moores, G. D., and Devonshire, A. L. (1998). Intensification of insecticide resistance in UK field populations of the peach-potato aphid, *Myzus persicae* (Hemiptera: Aphididae) in 1996. *Bulletin of Entomological Research* **88**, 127-130.

Foster, S. P., Harrington, R., Devonshire, A. L., Denholm, I., Clark, S. J., and Mugglestone, M. A. (1997). Evidence for a possible fitness trade-off between insecticide resistance and the low temperature movement that is essential for survival of UK populations of *Myzus persicae* (Hemiptera: Aphididae). *Bulletin of Entomological Research* **87**, 573-579.

Foster, S. P., Harrington, R., Devonshire, A. L., Denholm, I., Devine, G. J., Kenward, M. G., and Bale, J. S. (1996). Comparative survival of insecticide-susceptible and resistant peach- potato aphids, *Myzus persicae* (Sulzer) (Hemiptera, Aphididae), in low-temperature field trials. *Bulletin of Entomological Research* **86**, 17-27.

Foster, S. P., and Harris, M. O. (1997). Behavioral manipulation methods for insect pest-management. *Annual Review of Entomology* **42**, 123-146.

Foster, S. P., Woodcock, C. M., Williamson, M. S., Devonshire, A. L., Denholm, I., and Thompson, R. (1999). Reduced alarm response by peach-potato aphids, *Myzus persicae* (Hemiptera: Aphididae), with knock-down resistance to insecticides (kdr) may impose a fitness cost through increased vulnerability to natural enemies. *Bulletin of Entomological Research* **89**, 133-138.

Fowler, J., Cohen, L., and Jarvis, P. (1998). "Practical statistics for field biology," Second/Ed. Wiley J. and Sons, Chichester.

Fransen, J. J., and van Lenteren, J. C. (1993). Host selection and survival of the parasitoid *Encarsia formosa* an greenhouse-whitefly, *Trialeurodes*

vaporariorum, in the presence of host infected with the fungus *Ashersonia aleyrodis*. *Entomologia Experimentalis Et Applicata* **69**, 239-249.

Fransen, J. J., and van Lenteren, J. C. (1994). Survival of the parasitoid *Encarsia formosa* after treatment of parasitised greenhouse whitefly larvae with fungal spores of *Ashersonia aleyrodis*. *Entomologia Experimentalis Et Applicata* **71**, 235-243.

Furlong, M. J., and Pell, J. K. (1996). Interactions between the fungal entomopathogen *Zoophthora radicans* Brefeld (Entomophthorales) and 2 hymenopteran parasitoids attacking the diamonback moth, *Plutella xylostela* L. *Journal of Invertebrate Pathology* **68**, 15-21.

Furlong, M. J., Pell, J. K., and Reddy, G. V. P. (1997). Premortality effects of *Zoophthora radicans* infection in *Plutella xylostella*. *Journal of Invertebrate Pathology* **70**, 214-220.

Gams, W. (1971). "Cephalosporium-*artige* Schimmelpilze (*Hyphomycetes*)," Gustav Fischer Verlag, Stuttgart.

Gams, W., and Van Zaayen, A. (1982). Contribution to the taxonomy and the pathogenicity of fungicolous *Verticillium* species. I. Taxonomy. *Netherlands Journal of Plant Pathology* **88**, 57-78.

Gardner, W. A., and Kinard, D. J. (1998). *In vitro* germination and growth response of two entomogenous fungi to imidacloprid. *Journal of Entomological Science* **33**, 322-324.

Garret, A., Kerlan, C., and Thomas, D. (1996). Ultrastructural study of acquisition and retention of potato leafroll luteovirus in the alimentary canal of its aphid vector, *Myzus persicae* (Sulz.). *Archives of Virology* **141**, 1279-1292.

Georghiou, G. P. (1994). Principles of insecticide resistance management. *Phytoprotection* **75**, 51-59.

Gibson, R. W., and Pickett, J. A. (1983). Wild potato repels aphids by release of aphid alarm pheromone. *Nature* **302**, 608-609.

Gibson, R. W., and Rice, A. D. (1988). Modifying aphid behaviour. *In* "Aphids their biology, natural enemies and control." (A. K. Minks and P. Harrewijn, eds.), Vol. B. Elsevier, Amsterdam.

Goldsmith, T. H., and Bernard, G. D. (1974). The visual system of insects. *In* "The Physiology of Insecta" (M. Rockstein, ed.), Vol. 2, pp. 166-258. Academic Press, New York, London.

Grasswitz, T. R. (1998). Effect of adult experience on the host-location behavior of the aphid parasitoid *Aphidius colemani* Viereck (Hymenoptera: Aphidiidae). *Biological Control* **12**, 177-181.

Griffiths, D. C., Greenway, A. R., and Lloyd, S. L. (1978). The influence of repellent materials and aphid extracts on settling behaviour and the larviposition of *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *Buuletin of Entomological Research* **68**, 613-619.

Griffiths, D. C., and Pickett, J. A. (1980). A potential application of aphid alarm pheromones. *Entomologia experimentalis et applicata* **27**, 199-201.

Griffiths, D. C., and Pickett, J. A. (1987). Novel chemicals and their formulation for aphid control. *In* "14th International Symposium on Controlled release of bioactive materials" (P. I. Lee and B. A. Leonhardt, eds.). The Controled Release Society, Inc., Torondo.

Gullan, P. J., and Cranston, P. S. (1994). "The insects: an outline of entomology," Chapman & Hall, London.

Gutiérrez, C., Fereres, A., Reina, M., Cabrera, R., and GonzalezColoma, A. (1997). Behavioral and sublethal effects of structurally related lower terpenes on *Myzus persicae. Journal of Chemical Ecology* **23**, 1641-1650.

Hader, D. P. (1994). Real-time tracking of microorganisms. *Binary-Computing in Microbiology* **6**, 81-86.

Hajek, A. E. (1989). Food consumrtion by *Lymantria dispar* (Lepidoptera: Lymantriae) larvae infected with *Entomophaga maimaiga* (Zygomycetes: Entomophthorales). *Environmetal Entomology* **18**, 723-727.

Hajek, A. E., and St Leger, R. J. (1994). Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology* **39**, 293-322.

Hall, R. A. (1976a). A bioassay of the pathogenicity of the *Verticillium lecanii* conidiospores on the aphid, *Macrosiphoniella sanborni*. *Journal of invertebrate pathology* **27**, 41-48.

Hall, R. A. (1976b). *Verticillium lecanii on* the aphid *Macrosiphoniella sanborni*. *Journal of Invertebrate Pathology* **28**, 389-391.

Hall, R. A. (1977). The potential of the fungus *Verticillium lecanii* as a microbial control agent of glasshouse aphid pests, University of Southampton, Southampton.

Hall, R. A. (1979). Pathogenicity of *Verticillium lecanii* conidia and blastospores against the aphid, *Macrosiphonella sanborni*. *Entomophaga* **24**, 191-198.

Hall, R. A. (1981). The fungus *Verticillium lecanii* as a microbial insecticide against aphids and scales. *In* "Microbial control of pests and plant diseases 1970-1980." (H. D. Burges, ed.). Academic Press.

Hall, R. A. (1982). Deuteromycetes: virulence and bioassay design. *In* "Proceeding of the 3rd Internatinal Colloquium of Inveretbrate Pathology", pp. 191-196.

Hall, R. A. (1984). Epizootic potential for aphids of different isolates of the fungus *Verticillium lecanii*. *Entomophaga* **29**, 311-321.

Hall, R. A., and Burges, H. D. (1979). Control of aphids in glasshouse with the fungus *Verticillium lecanii*. *Annals of applied biology* **93**, 235-246.

Hall, R. A., and Papierok, B. (1982). Fungi as biological control agents of athropods of agricultural and medical importance. *Parasitology* **84**, 205-240.

Hallsworth, J. E., and Magan, N. (1994). Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of conidia of 3 entomopathogenic fungi. *Microbiology-Uk* **140**, 2705-2713.

Hallsworth, J. E., and Magan, N. (1995). Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. *Microbiology-Uk* **141**, 1109-1115.

Hardie, J., and Young, S. (1997). Aphid flight-track analysis in three dimensions using video techniques. *Physiological Entomology* **22**, 116-122.

Harper, A. M. (1958). Notes on behaviour of *Pemphigus betae* Doane (Homoptera: Aphididae) infected with *Entomophthora aphidis* Hoffm. *Canadian Entomology* **90**, 439-440.

Hassan, A. E. M., and Charnley, A. K. (1989). Ultrastructural-study of the penetration by *Metarhizium anisopliae* through dimilin-affected cuticle of *Manduca sexta*. *Journal of Invertebrate Pathology* **54**, 117-124.

Hassan, A. E. M., Dillon, R. J., and Charnley, A. K. (1989). Influence of accelerated germination of conidia on the pathogenicity of *Metarhizium anisopliae* for *Manduca sexta*. *Journal of Invertebrate Pathology* **54**, 277-279.

Haynes, K. F. (1988). Sublethal effects of neurotoxic insecticides on insect behaviour. *Annual Review of Entomology* **33**, 149-168.

Heale, J. B. (1988). The potential impact of fungal genetics and molecular biology on biological control. with particular reference to entomopathogens. *In* "Fungi in biological control systems" (M. N. Burge, ed.). Manchester University Press.

Heale, J. B., Isaac, J. E., and Chandler, D. (1989). Prospects for strain improvement in entomopathogenic fungi. *Pesticide Science* **26**, 79-92.

Hill, D. (1987). "Agricultural insects pests of temperate regions and their control," Cambridge Univeristy Press, London.

Hori, M., and Harada, H. (1995). Screening plants resistant to green peach aphid, *Myzus persicae* (Sulzer) (Homoptera, Aphididae). *Applied Entomology and Zoology* **30**, 246-249.

Horton, D. R., and Moore, J. (1993). Behavioral effects of parasites and pathogens in insect hosts. *In* "Parasites and Pathogens of Insects" (N. E. Beckage, S. N. Thompson and B. A. Federici, eds.), Vol. 1: Parasites. Academic Press.

Hoy, J. B., Globus, P. A., and Norman, K. D. (1983). Electronic tracking and recording-system for behavioural observations, with application to toxicology and pheromone assay. *Journal of Economic Entomology* **76**, 678-680.

Hoy, J. B., Koehler, P. G., and Patterson, R. S. (1996). A microcomputer-based system for real-time analysis of animal. *Journal of Neuroscience Methods* **64**, 157-161.

Hoy, J. B., Porter, S. D., and Koehler, P. G. (1997). A microcomputer-based activity meter for multiple animals. *Journal of Neuroscience Methods* **72**, 183-188.

Inglis, G. D., Johnson, D. L., and Goettel, M. S. (1996). Effects of temperature and thermoregulation on mycosis by *Beauveria bassiana* in grasshoppers. *Biological Control* 7, 131-139.

Jackson, C. W., Heale, B. J., and Hall, R. A. (1985). Traits associated with virulence to the aphid *Macrosiphoniella sanborni* in 18 isolates of *Verticillium lecanii*. *Annals of Applied Biology* **106**, 39-48.

Jaronski, S. T., and Goettel, M. S. (1997). Development of *Beauveria bassiana* for control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada*, 225-237.

Jenkins, N. E., and Thomas, M. B. (1996). Effect of formulation and application method on the efficacy of aerial and submerged conidia of *Metarhizium flavoviride* for locust and grasshopper control. *Pesticide Science* **46**, 299-306.

Joshi, L., Charnley, A. K., Arnold, G., Brain, P., and Bateman, R. (1992). Synergism between the entomopathogenic fungus *Metarhizium spp.* and the benzoyl phenyl urea insecticide teflubensuron, against the desert locust *Schistocerca gregaria*. *In* "Brighton Crop Protection Conference", pp. 369-374, Brighton.

Jun, Y., Bridge, P. D., and Evans, H. C. (1991). An integrated approach to the taxonomy of the genus *Verticillium*. *Journal of General Microbiology* **137**, 1437-1444.

Kaakeh, W., Reid, B. L., Bohnert, T. J., and Bennett, G. W. (1997). Toxicity of imidacloprid in the German cockroach (Dictyoptera: Blattellidae), and the synergism between imidacloprid and *Metarhizium anisopliae* (Imperfect Fungi: Hyphomycetes). *Journal of Economic Entomology* **90**, 473-482.

Kenedy, J. S., Ibbotson, A., and Booth, C. O. (1950). The distribution of aphid infestation in relation to leaf age. I. *Myzus persicae* (Sulz.) and *Aphis fabae* Scop. on spindle trees and sugarbeet plants. *Annals of applied biology* **37**, 651-679.

Klingauf, F. (1981). Interrelations between pests and climatic factors. *In* "Food - Climate Interactions" (W. Bach, J. Pankrath and S. H. Schneider, eds.), pp. 285-301. Reidel D. Publishing company, Dordrecht.

Klingauf, F. A. (1987a). Feeding, adaptation and excretion. *In* "Aphids their biology, natural enemies and control" (A. K. Minks and P. Harrewijn, eds.), Vol. A, pp. 225-253. Elsevier, Amsterdam.

Klingauf, F. A. (1987b). Host plant finding and acceptance. *In* "Aphids their biology, natural enemies and control" (A. K. Minks and P. Harrewijn, eds.), Vol. A, pp. 209-223. Elsevier, Amsterdam.

Kouvelis, V. N., Zare, R., Bridge, P. D., and Typas, M. A. (1999). Differentiation of mitochondrial subgroups in the *Verticillium lecanii* species complex. *Letters in Applied Microbiology* **28**, 263-268.

Krasnoff, S. B., Watson, D. W., Gibson, D. M., and Kwan, E. C. (1995). Behavioural effects of the entomopathogenic fungus, *Entomophthora muscae* on its host *Musca domestica*: postural changes in dying host and gated pattern of mortality. *J. Insect Physiol.* **41**, 895-903.

Krens, F. A., Verhoeven, H. A., VanTunen, A. J., and Hall, R. D. (1998). The use of an automated cell tracking system to identify specific cell types competent for regeneration and transformation. *In Vitro Cellular & Developmental Biology-Plant* **34**, 81-86.

Lacey, L. A., and Goettel, M. S. (1995). Current developments in microbial control of insects pests and prospects for the early 21st century. *Entomophaga* **40**, 3-27.

Lagnaoui, A., and Radcliffe, E. B. (1998). Potato fungicides interfere with entomopathogenic fungi impacting population dynamics of green peach aphid. *American Potato Journal* **75**, 19-25.

Latgé, J. P., and Papierok, B. (1988). Aphid pathogens. *In* "Aphids their biology, natural enemies and control" (A. K. Minks and P. Harrewijn, eds.), Vol. A, pp. 323-335. Elsevier, Amsterdam.

Lees, A. D. (1966). The control of polymorphism in aphids. *Advances in Insect Physiology* **3**, 207-277.

Lees, A. D. (1971). The relevance of action spectra in study of insect photoperiodism. *In* "Biochronometry" (M. Menaker, ed.), pp. 372-380. National Academy of Science, Washington.

Leicht, W. (1993). Imicacloprid - a chloronicotyl insecticide. *Pesticide Outlook* **4**, 17-24.

Lemon, R. W. (1994). Insecticide Resistance. *Journal of Agricultural Science* **122**, 329-333.

Leszczynski, B., Tjallingii, W. F., Dixon, A. F. G., and Swiderski, R. (1995). Effect of methoxyphenols on grain aphid feeding behaviour. *Entomologia Experimentalis Et Applicata* **76**, 157-162.

Liu, M. Y., and Casida, J. E. (1993). High-affinity binding of [H-3] Imidacloprid in the insect acetylcholine-receptor. *Pesticide Biochemistry and Physiology* **46**, 40-46.

Liu, M. Y., Lanford, J., and Casida, J. E. (1993). Relevance of [H-3] Imidacloprid binding-site in house-fly head acetylcholine-receptor to insecticidal activity of 2-nitromethylene- imidazolidines and 2-nitroimino-imidazolidines. *Pesticide Biochemistry and Physiology* **46**, 200-206.

Loebenstein, G., and Raccah, B. (1980). Control of non-persistently transmitted aphid-borne viruses. *Phytoparasitica* **8**, 221-235.

Lowe, H. J. B., and Taylor, L. R. (1964). Population parameters, wing production and behaviour in red and green *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae). *Entomologia Experimentalis Et Applicata* **7**, 287-295.

Lowery, D. T., Eastwell, K. C., and Smirle, M. J. (1997). Neem seed oil inhibits aphid transmission of potato virus Y to pepper. *Annals of Applied Biology* **130**, 217-225.

Lowery, D. T., and Isman, M. B. (1996). Inhibition of aphid (Homoptera, Aphididae) reproduction by Neem seed oil and azadirachtin. *Journal of Economic Entomology* **89**, 602-607.

Majchrowicz, I., and Poprawski, T. J. (1993). Effects *in vitro* of 9 fungicides on growth of entomopathogenic fungi. *Biocontrol Science and Technology* **3**, 321-336.

Martinez Torres, D., Chandre, F., Williamson, M. S., Darriet, F., Berge, J. B., Devonshire, A. L., Guillet, P., Pasteur, N., and Pauron, D. (1998). Molecular

characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae* s.s. *Insect Molecular Biology* **7**, 179-184.

Matsuka, M., and Mittler, T. E. (1978). Enhancement of alatae production by an aphid, *Myzus persicae*, in response to increases in daylength. *Bulletin of the Faculty of Agriculture of Tamagawa University Tokyo* **18**, 1-7.

Matthews, R. W., and Matthews, J. R. (1978). "Insect behaviour," John Wiley & sons, New York.

May, B., and Holbrook, F. R. (1978). Absence of genetic variability in the green peach aphid, *Myzus persicae* (Hemiptera: Aphididae). *Annals of Entomological Society of America* **71**, 809-812.

McCoy, C. W., Samson, R. A., and Boucias, D. G. (1988). Entomogenous fungi. *In* "CRC Handbook of Natural Pesticides. Microbial Insecticides, Part A. Entomogenous Protozoa and Fungi" (C. M. Ignoffo, ed.), Vol. 5.

McKinlay, R. G., Spaull, A. M., and Straub, R. W. (1992). Pests of Solanaceous Crops. *In* "Vegetables crop pests." (R. G. McKinlay, ed.). Macmilan press.

McLean, D. L., and Kinsey, M. G. (1964). A technique for electronically recording aphid feeding behaviour. *Nature* **202**, 1358-1359.

Menzel, R. (1975). Colour receptors in insects. *In* "The compound eye and vision of insects" (G. A. Horridge, ed.), pp. 121-153. Clarendon Press, Oxford.

Metcalf, R. L. (1989). Inects resistance to insecticides. *Pesticide science* **26**, 333-358.

Miles (1987). Feeding process of Aphidoidea in relation to effects on their food plants. *In* "Aphids their biology, natural enemies and control" (A. K. Minks and P. Harrewijn, eds.), Vol. 2A, pp. 321-339. Elsevier, Amsterdam.

Milner, R. J. (1997). Prospects for biopesticides for aphid control. *Entomophaga* **42**, 227-239.

Milner, R. J., and Lutton, G. G. (1986). Dependence of *Verticillium lecanii* (Fungi: Hyphomycetes) on high humidities for infection and sporulation using *Myzus persicae* (Homoptera: Aphididae) as host. *Environmental Entomology* **15**, 380-382.

Minchella, D. J. (1985). Host life-history variation in response to parasitism. *Parasitology* **90**, 205-216.

Molyneux, D. H., and Jeffries, D. (1986). Feeding behaviour of pathogen-infected vectors. *Parasitology* **92**, 721-736.

Montgomery, M. E., and Nault, L. R. (1977). Coparative response of aphids to the alarm pheromone, (*E*)-ß-fernesene. *Entomologia experimentalis et applicata* **22**, 236-242.

Moore, D., Reed, M., Le Patourel, G., Abraham, Y. J., and Prior, C. (1992). Reduction of feeding by the desert locust, *Schistocerca gregaria*, after infection with *Metarhizium flavoviride*. *Journal of Invertebrate Pathology* **60**, 304-307.

Moores, G. D., Devine, G. J., and Devonshire, A. L. (1994). Insecticide-insensitive acetylcholinesterase can enhance esterase-based resistance in *Myzus persicae* and *Myzus nicotianae*. *Pesticide Biochemistry and Physiology* **49**, 114-120.

Moorhouse, E. R. (1990). The potential of the entomogenous fungus *Metarhizium anisopliae* as a microbial control agent of the black vine weevil *Otiorhynchus sulcatus*, University of Bath, Bath.

Moorhouse, E. R., Gillespie, A. T., Sellers, E. K., and Charnley, A. K. (1992). Influence of fungicides and insecticides on the entomogenous fungus *Metarhisium anisopliae*, a pathogen of the vine weevil, *Otiohynchus sulcatus*. *Biocontrol Science and Technology* **2**, 49-58.

Nauen, R. (1995). Behavior modifying effects of low systemic concentrations of Imidacloprid on *Myzus persicae* with special reference to an antifeeding response. *Pesticide Science* **44**, 145-153.

Nauen, R., and Elbert, A. (1994). Effect of imidacloprid on aphids after seed treatment of cotton in laboratory and greenhouse experiments. *Pflanzenschutz-Nachrichten Bayer* **47**, 177.

Nauen, R., and Elbert, A. (1997). Apparent tolerance of a field-collected strain of *Myzus nicotianae* to imidacloprid due to strong antifeeding responses. *Pesticide Science* **49**, 252-258.

Nauen, R., Hungenberg, H., Tollo, B., Tietjen, K., and Elbert, A. (1998a). Antifeedant effect, biological efficacy and high affinity binding of imidacloprid to acetylcholine receptors in *Myzus persicae* and *Myzus nicotianae*. *Pesticide Science* **53**, 133-140.

Nauen, R., Koob, B., and Elbert, A. (1998b). Antifeedant effects of sublethal dosages of imidacloprid on *Bemisia tabaci*. *Entomologia Experimentalis Et Applicata* **88**, 287-293.

Nauen, R., Strobel, J., Tietjen, K., Otsu, Y., Erdelen, C., and Elbert, A. (1996). Aphicidal activity of imidacloprid against a tobacco feeding strain of *Myzus persicae* (Homoptera, Aphididae) from Japan closely-related to *Myzus nicotianae* and highly resistant to carbamates and organophosphates. *Bulletin of Entomological Research* **86**, 165-171.

Nauen, R., Tietjen, K., Wagner, K., and Elbert, A. (1998c). Efficacy of plant metabolites of imidacloprid against *Myzus persicae* and *Aphis gossypii* (Homoptera: Aphididae). *Pesticide Science* **52**, 53-57.

Nisbet, A. J., Woodford, J. A. T., and Strang, R. H. C. (1994). The effects of azadirachtin-treated diets on the feeding-behavior and fecundity of the peach-potato aphid, *Myzus persicae*. *Entomologia Experimentalis Et Applicata* **71**, 65-72.

Nisbet, A. J., Woodford, J. A. T., Strang, R. H. C., and Connolly, J. D. (1993). Systemic antifeedant effects of azadirachtin on the peach-potato aphid *Myzus persicae*. *Entomologia Experimentalis Et Applicata* **68**, 87-98.

Pickett, J. A. (1991). Pheromones - will their promise in insect pest-control ever be achieved. *Bulletin of Entomological Research* **81**, 229-232.

Pickett, J. A., Wadhams, L. J., and Woodcock, C. M. (1989). Chemical ecology and pest management: Some recent insights. *Insect Sci. Applic.* **10**, 741-750.

Pickett, J. A., Wadhams, L. J., Woodcock, C. M., and Hardie, J. (1992). The chemical ecology of aphids. *Annual Review of Entomology* **37**, 67-90.

Pollard, D. G. (1973). Plant penetration by feeding aphids (Hemiptera, Aphidoidea): a review. *Bulletin of Entomological Research* **62**, 631-714.

Poprawski, T. J., and Majchrowicz, I. (1995). Effects of herbicides on *in vitro* vegetative growth and sporulation of entomopathogenic fungi. *Crop Protection* **14**, 81-87.

Potter, C. (1952). An improved laboratory apparatus for applying direct sprays and surface films, with data on the electrostatic charge on atomised fluids. *Annals of Applied Biology* **39**, 1-28.

Powell, G. (1993). The effect of pre-acquisition starvation on aphid transmission of potyviruses during observed and electrically recorded stylet penetrations. *Entomologia Experimentalis Et Applicata* **66**, 255-260.

Powell, G., Hardie, J., and Pickett, J. A. (1995a). Behavioral evidence for detection of the repellent polygodial by aphid antennal tip sensilla. *Physiological Entomology* **20**, 141-146.

Powell, G., Hardie, J., and Pickett, J. A. (1995b). Responses of *Myzus persicae* to the repellent polygodial in choice and no-choice video assays with young and mature leaf tissue. *Entomologia Experimentalis et Applicata* **74**, 91-94.

Powell, G., Hardie, J., and Pickett, J. A. (1996). Effects of the repellent polygodial on stylet penetration behaviour and non-persistent transmission of plant viruses by aphids. *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie* **120**, 241-243.

Powell, G., Hardie, J., and Pickett, J. A. (1998). The effects of antifeedant compounds and mineral oil on stylet penetration and transmission of potato virus Y by *Myzus persicae* (Sulz.) (Hom., Aphididae). *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie* **122**, 331-333.

Powell, W., Willding, N., Brobyn, P. J., and Clark, S. J. (1986). Interference between parasitoids (Hym.: Aphidiidae) and fungi (Entomophthorales) attacking cereal aphids. *Entomophaga* **31**, 293-302.

Prior, C. (1992). Discovery and characterisation of fungal pathogens for locust and grasshopper control. *In* "Biological control of locusts and grasshoppers" (C. J. Lomer and C. Prior, eds.), pp. 159-180. CAB, Wallingford, Oxon, UK.

Quicke, D. L. J. (1997). "Parasitic Wasps," Chapman and Hall.

Quintela, E. D., and McCoy, C. W. (1997a). Effects of imidacloprid on development, mobility, and survival of first instars of *Diaprepes abbreviatus* (Coleoptera: Curculionidae). *Journal of Economic Entomology* **90**, 988-995.

Quintela, E. D., and McCoy, C. W. (1997b). Pathogenicity enhancement of *Metarhizium anisopliae* and *Beauveria bassiana* to first instars of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) with sublethal doses of imidacloprid. *Environmental Entomology* **26**, 1173-1182.

Quintela, E. D., and McCoy, C. W. (1998a). Conidial attachment of *Metarhizium anisopliae* and *Beauveria bassiana* to the larval cuticle of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) treated with imidacloprid. *Journal of Invertebrate Pathology* **72**, 220-230.

Quintela, E. D., and McCoy, C. W. (1998b). Synergistic effect of imidacloprid and two entomopathogenic fungi on the behaviour and survival of larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in soil. *Journal of Economic Entomology* **91**, 110-122.

Ramakers, P. M. J. (1988). Biological control in greenhouses. *In* "Aphids their biology, natural enemies and control" (A. K. Minks and P. Harrewijn, eds.), Vol. C, pp. 199-208. Elsevier, Amsterdam.

Rice, A. D., Gibson, R. W., and Stribley, M. F. (1983). Effects of deltamethrin on walking, flight and potato virus Y-transmission by pyrethroid-resistant *Myzus persicae*. *Annals of Applied Biology* **102**.

Roberts, D. W. (1981). Toxins of entomopathogenic fungi. *In* "Microbial control of pests and plant diseases 1970-1980." (H. D. Burges, ed.). Acad. Press.

Rockwood, L. P. (1950). Entomogenous fungi of the family Entomophthoraceae in the Pacific Northwest. *Journal of Economic Entomology* **43**, 704-707.

Rowe, R. C. (1995). Recent progress in understanding relationships between *Verticillium* species and subspecific groups. *Phytoparasitica* **23**, 31-38.

Saito, T., and Yabuta, M. (1996). Laboratory studies on effect of pesticides on entomopathogenic fungus, *Verticillium lecanii*. *Japanese Journal of Applied Entomology and Zoology* **40**, 71-76.

Samson, R. A. (1981). Identification: Entomopathogenic Deuteromucetes. *In* "Microbial control of pests and plant diseases 1970-1980." (H. D. Burges, ed.). Acad. Press.

Samson, R. A., Evans, H. C., and Latgé, J. P. (1988). "Atlas of Entomopathogenic Fungi," Springer-Verlag, Berlin.

Samuels, R. I., Reynolds, S. E., and Charnley, A. K. (1988). Calcium-channel activation of insect muscle by destruxins, insecticidal compounds produced by the entomopathogenic fungus *Metarhizium anisopliae*. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **90**, 403-412.

Sanyang, S., and VanEmden, H. F. (1996). The combined effects of the fungus *Metarhizium flavoviride* Gams & Rozsypal and the insecticide cypermethrin on *Locusta migratoria* migratorioides (Reiche & Fairmaire) in the laboratory. *International Journal of Pest Management* **42**, 183-187.

Saunders, D. S. (1982). "Insect Clocks," Second/Ed. Pergamon Press.

Schnorbach, H. J. (1983). Der Einfluss von Besiedlungs und Einstecherhalten auf Beginn und Verlauf der Nahrungsaufnahme bei Blattlausgruppen an Wirtspflanze und küstlicher Diät (Aphididea, Homoptera), University of Bonn.

Schreiter, G., Butt, T. M., Beckett, A., and Moritz, G. (1995). Electron microscope studies of infection by *Verticillium lecanii* (Zimm.) Viégas of wetsren flower thrips. *In* "Thrips biology and management" (P. B. L. e. al., ed.), pp. 297-304. Plenum Press, New York.

Schreiter, G., Butt, T. M., Beckett, A., Vestergaard, S., and Moritz, G. (1994). Invasion and development of *Verticillium lecanii* in the western flower thrips, *Frankiniella occidentalis*. *Mycological research* **98**, 1025-1034.

Sherwood, M. H., Greenway, A. R., and Griffiths, D. C. (1981). Responses of *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) to plants treated with fatty acids. *Bulletin of Entomological Research* **71**, 133-136.

Sitch, J. C., and Jackson, C. W. (1997). Pre-penetration events affecting host specificity of *Verticillium lecanii*. *Mycological Research* **101**, 535-541.

Sokal, R. R., and Rohlf, F. J. (1995). "Biometry: The principles and practice of statistics in biological research," Third/Ed. W. H. Freeman and Company, New York.

Sopp, P. I., Gillespie, A. T., and Palmer, A. (1990). Comparison of ultra-low-volume electrostatic and high volume hudravlic application of *Verticillium lecanii* for aphid control on chrysanthemums. *Crop Protection* **9**, 177-184.

St Leger, R. J., Joshi, L., Bidochka, M. J., and Roberts, D. W. (1996). Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 6349-6354.

Starý, P. (1988). Natural enemies. *In* "Aphids their biology, natural enemies and control" (A. K. Minks and P. Harrewijn, eds.), Vol. B. Elsevier, Amsterdam.

Steenberg, T., and Humber, R. A. (1999). Entomopathogenic potential of *Verticillium* and *Acremonium* species (Deuteromycotina: Hyphomycetes). *Journal of Invertebrate Pathology* **73**, 309-314.

Steinkraus, D. C., and Tugwell, N. P. (1997). *Beauveria bassiana* (Deuteromycotina: Moniliales) effects on *Lygus lineolaris* (Hemiptera: Miridae). *Journal of Entomological Science* **32**, 79-90.

Suomalainen, E., Saura, A., Lokki, J., and Teeri, T. (1980). Genetic polymorphism in parthenogenetic animals. Pt. 9. Absence of variation within parthenogenetic aphid clones. *Theoretical and Applied Genetics* **57**, 129-132.

Sutherland, O. R. W. (1969). The role of crowding in the production of winged forms by two strains of the pea aphids, *Acyrthosiphon pisum*. *Journal of Insect Physiology* **15**, 2179-2200.

Sylvester, E. (1954). Insectary life history and apterous instar morphology of *Myzus persicae* (Sulzer). *Annals of Entomological Society of America* **47**, 397-406.

Tanada, Y., and Kaya, H. (1993). "Insect Pathology," Academic Press.

Tjallingii, W. F. (1985). Electrical nature of recorded signals during stylet penetration by aphids. *Entomologia Experimentalis et Applicata* **38**, 178-186.

Tjallingii, W. F., and Hogen Esch, T., (1993). Fine structure of aphids stylet routes in plant tissues in correlation with EPG signals. *Physiological Entomology* **18**, 317-328.

Tomiuk, J., and Wöhrmann, K. (1981). Changes of the genotype frequencies at the MDH-Locus in populations of *Macrosirphum rosae* (L.) (Hemiptera, Aphididae). *Biol. Zentralbl.* **100**, 631-640.

Tourtellot, M. K., Colins, R. D., and Bell, W. J. (1991). The problem of movelength and turn definition in analysis of orientation data. *Journal of theoretical biology* **150**, 287-297.

Van Driesche, R. G., and Bellows, T. S. J. (1996). "Biological Control," Chapman and Hall, New York.

van den Heuvel, J. F. J. M., Hogenhout, S. A., Verbeek, M., and van der Wilk, F. (1998). *Azadirachta indica* metabolites interfere with the host-endosymbiont relationship and inhibit the transmission of potato leafroll virus by *Myzus persicae*. *Entomologia Experimentalis Et Applicata* **86**, 253-260.

Varley, M. J., Copland, M. J. W., Wratten, S. D., and Bowie, M. H. (1994). Parasites and predators. *In* "Video techniques in animal ecology and behaviour" (S. D. Wratten, ed.). Chaman and Hall, London.

Vatine, J. J., Ratner, A., Dvorkin, M., and Seltzer, Z. (1998). A novel computerized system for analyzing motor and social behavior in groups of animals. *Journal of Neuroscience Methods* **85**, 1-11.

Vet, L. E. M., and Groenewold, A. W. (1990). Semiochemicals and learning in parasitoids. *Journal of Chemical Ecology* **16**, 3119-3135.

Viégas, A. P. (1939). Um amigo de fazendeiro *Verticillium lecanii* n. com. O causador do halo branco do *Coccus viridis*. *Revista do Institute de Cafe Estado Sao Paulo* **14**, 754-772.

Voss, G. (1988). Insecticide/acaricide resistance: industry's efforts and plans to cope. *Pesticide Science* **23**, 149-205.

Watson, D. W., Mullens, B. A., and Petersen, J. J. (1993). Behavioural fever response of *Musca domestica* (Diptera: Muscidae) to infection by *Entomophtora*

muscae (Zygomycetes: Entomophthorales). *Journal of Invertebrate Pathology* **61**, 10-16.

Way, M. J. (1967). Intra-specific mechanism with special reference to aphid population. *Symposia of the Royal Entomological Society of London* **4**, 18-36.

Wellings, P. W., Lether, S. R., and Dixon, G. A. F. (1980). Seasonal variation in the reproductive potential: a programmed feature of aphids life cycles. *Journal of Animal Ecology* **49**, 975-985.

Williams, K. P. (1995). Effects of insecticides on susceptibility to *Verticillium lecanii* amongst clones of *Myzus persicae*. M.Sc. in Crop Protection, University of Bath.

Wohlers, P. (1981). Aphid avoidance of plants contaminated with alarm pheromone (*E*)-β-farnesene. *Zeitschrift für Angewandte Entomologie* **92**, 329-336.

Yokomi, R. K., and Gottwald, T. R. (1988). Virulence of *Verticillium lecanii* isolates in aphids determined by detached-leaf bioassay. *Journal of Invertebrate Pathology* **51**, 250-258.

Zimmermann, A. (1898). 'A fungus disease of *Lecanium viride*'. Over eene schimmelepidemie der groene Luizen. *Korte Berichten Uit's Lands plantentuin* **9**, 240-243.

Appendix 1 - Genstat programs

Program for LD_{50}

```
units [20]                          - Defines default length for variates
scalar t; 4                         - Defines the number of trials for each variate
factor [levels=#t] trial            - Defines factor containing trial levels
read trial,conc,total,kill          - Inputs data in the format specified
1  0         50  1                  - First number is a numerical identifier for each trial
1  1000      50  3
1  10000     50  6
1  100000    50  23
1  1000000   50  40
2  0         50  0
2  1000      50  2
2  10000     50  5
2  100000    50  29
2  1000000   50  34
3  0         50  3
3  1000      50  4
3  10000     50  6
3  100000    50  16
3  1000000   50  38
4  0         50  0
4  1000      50  0
4  10000     50  5
4  100000    50  19
4  1000000   50  38
:
"
 Calculate log 10 of conc
"
calculate [print=s] logconc = log10(conc)
"
 Run probitanalysis procedure treating whole set as one.
 not estimating mortality
"
probitan [print=m,s,e,c,f] kill; dose=logconc; nbin=total
"
 Define expression which antilogs LD50
"
expr LD50; !e(Ldose50=10**'LD50')
"
 Use rfunction to print out LD50 on original concentration scale with
 estimate of standard error.
"
rfunction [print=est,se; calc=LD50] Ldose50
"
 Now fit parallel lines. One for each trial
"
probitan [print=m,s,e,c,f; groups=trial] kill; dose=logconc;
nbin=total
"
 Works out LD50 with standard error for each trial. Performed in a
loop
"
```

```
for i = 1...t
    text dumt
    print [channel=dumt; squash=yes; iprint=*] 'LD50[',i,']';\
          field=4,1,0;dec=0; skip=0
    expr ld10[i]; !e(LDose50[i]=10**(#dumt))
    rfunction [print=est,se; calc=ld10[i]] LDose50[i]
endfor
"
 Now fit separate slope for each trial
"
probitan [print=m,s,e,c,f; groups=trial; sep=slope] kill;
dose=logconc;\
         nbin=total

for i = 1...t
    rfunction [print=est,se; calc=ld10[i]] LDose50[i]
endfor
"
 Repeat estimating mortality
"
probitan [mort=est;print=m,s,e,c,f] kill; dose=logconc; nbin=total
rfunction [print=est,se; calc=LD50] Ldose50

probitan [mort=est; print=m,s,e,c,f; groups=trial] kill;\
          dose=logconc; nbin=total

for i = 1...t
    rfunction [print=est,se; calc=ld10[i]] LDose50[i]
endfor

probitan [mort=est;print=m,s,e,c,f; groups=trial; sep=slope] kill;\
         logconc; total

for i = 1...t
    rfunction [print=est,se; calc=ld10[i]] LDose50[i]
endfor

stop
```

Program for Randomised Block Analysis

```
units [30]
open 'aphid.dat' ;2
factor [lev=3] block
factor [lev=2] treat
read [ch=2] treat, block, day2, day3, day4
calc d3sq = sqrt(day3)
"
```
Calculate square root (data transformation for day3)
```
"
print day3, d3sq, treat, block
block block
treat treat
anova [fprob=yes] d3sq
end
```

Program for Binomial Analysis

```
units [64]
factor [lev=2] side
factor [lev=11] treat
open 'depent-dose.dat2'; 2
read [ch=2] dose, dish, side, dead, n, treat
calc perc_dead = 100 * dead/n
"
```
Calculating the percentage of mortality
```
"
print treat, side, dead, n, perc_dead
model [distribution=binomial] dead; nbinomial=n
"
```
Assuming binomial distribution
```
"
fit [print=*] treat
add [print=*] side
add [print=acc; fprob=y] treat.side
tabulate [print=means; class= side] perc_dead
stop
```

Appendix 2 - File format, procedures and rules in computer tracking of aphid movement

Figure App-2.1. Tracking program Data Arrays and Analysis of the data by the 'Ant Data' Program and the resulting arrays.

The data produced by the tracking program are first stored in RAM memory. When the tracking secession is over the data are saved on the hard drive as Frame (or *Set-up file*) and Centroid text files. Frame file contains the frame number, the time that it was analysed and the number of blobs (individuals) on the screen. The Centroid File contains the *x*, *y* co-ordinates and the area of each blob (centre of gravity of individuals). Continues next page ...

Text file Formats

Frame file format

Frame number	Time (sec)	Number of individuals captured
0	0.0837518	5
1	0.326343	5
2	0.486356	5
3	0.646203	5
4	0.80634	6
5	0.966312	5
6	1.12631	5
7	1.28618	6
8	1.44616	5
9	1.6063	5
10	1.76623	7
11	1.92622	6
12	2.08612	5
13	2.24612	6
14	2.40617	7
15	2.56623	6
16	2.72625	6
17	2.88623	7
18	3.04612	6
19	3.20616	6
20	3.36645	6
21	3.5261	5
22	3.68604	6
.	..	.
.	..	.
.	..	.
19993	3199.77	11
19994	3199.93	10
19995	3200.09	9
19996	3200.25	9
19997	3200.41	9

Centroid file format

x co-ordinate	*y* co-ordinate	Area
582	172	112
293	140	111
426	134	132
484	118	40
450	475	115
507	470	34
573	180	32
582	172	110
293	140	111
427	134	146
484	118	38
582	172	113
293	140	113
426	134	135
485	118	41
450	475	110
507	470	32
573	179	27
.	..	.
.	..	.
.	..	.
574	180	29
582	172	111
294	140	119
427	134	138
485	119	47
509	469	32
574	179	29
582	172	114

'Behaviour' File Format

Identity N°	
0	0
5	1
1	1
3	1
4	1
2	1
0	0
5	1
1	1
3	1
4	1
2	1
0	0
2	1
0	0
5	1
1	1
.	.
.	.
.	.
4	1
2	1
0	0
5	1
2	1
1	1
3	1
4	1

The 'behaviour' text file associates certain *x*-*y* co-ordinates (tracked blobs) with an identity number and it is created during the analysis of the data (Ant Data Program). The blob identity is selected semi-automatically by following simple rules described in the 'Tracking aphids' section Chapter 2. Combining the 3 arrays the program is able to produce a complete file for a single individual. Basically all the blobs associated with the same identity N° are selected and are pasted in new file.

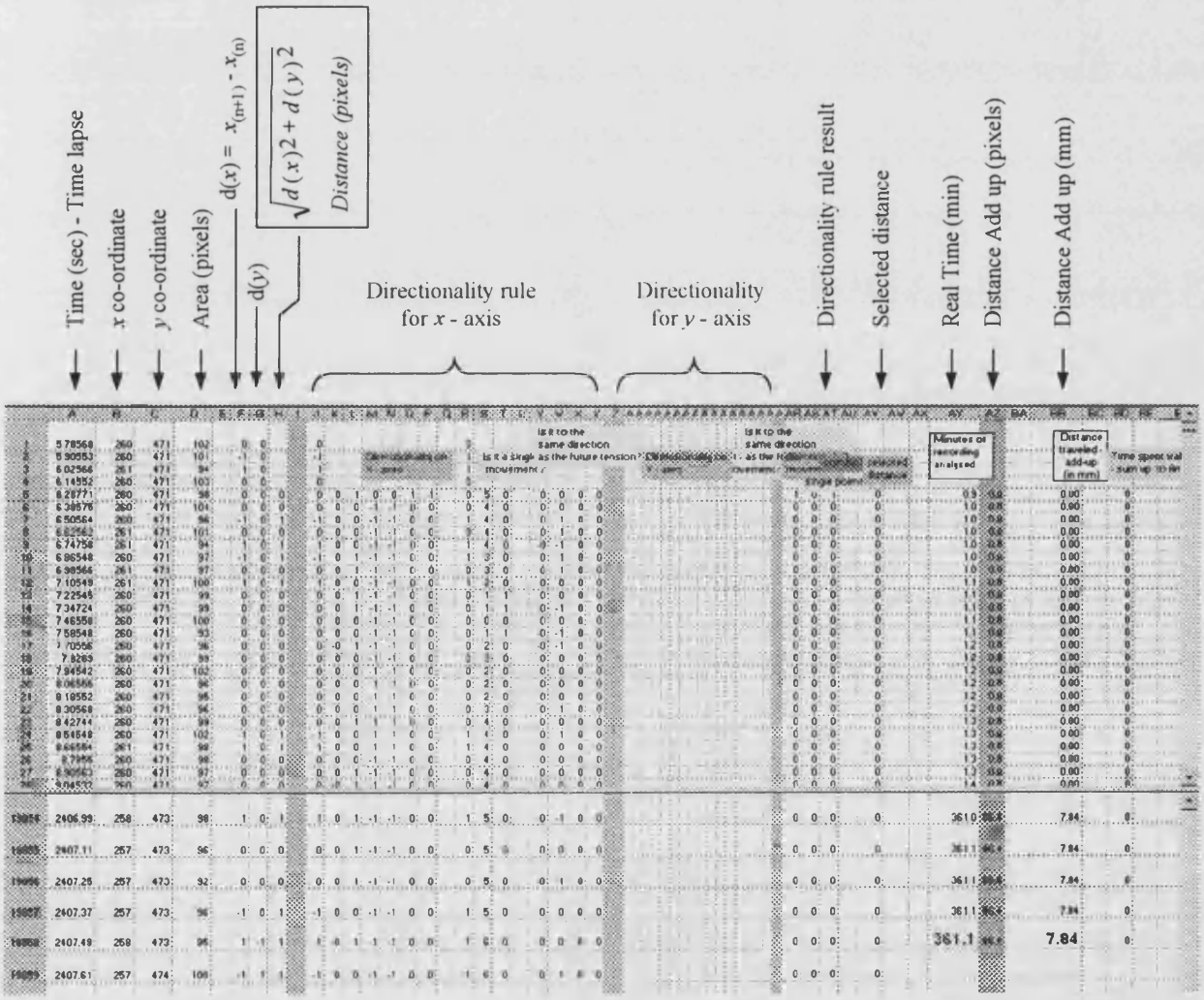
Data for a single individual (file format)

Time (sec)	*x* co-ordinate	*y* co-ordinate	Area
Agent number 3			
4.57132	480	85	119
4.73132	480	85	119
4.8913	480	85	118
5.05145	480	85	116
5.21143	480	85	120
5.37128	480	85	120
5.5313	480	85	122
5.69131	480	85	119
5.85126	480	85	122
6.01131	480	85	118
6.17134	480	85	119
6.33137	480	85	117
6.49135	480	85	121
6.65135	480	85	122
6.81126	480	85	113
6.97133	480	85	121
7.13132	480	85	119
7.2912	480	85	117
7.45121	480	85	117
7.61116	480	85	119
7.77143	480	85	121
7.93121	480	85	118
8.09114	480	85	120
8.25126	480	85	118
8.41125	480	85	121
..	.	.	..
..	.	.	..
..	.	.	..
3180.9	479	83	100
3181.06	478	83	98
3181.22	478	83	101
3181.38	478	83	97
3181.54	479	83	102
3181.7	479	83	104
3181.86	478	83	100
3182.02	479	83	97
3182.18	478	83	103

Figure App-2.2. Data analysis using Excel.

The text files with data processed with Ant Data Program for single individuals were inserted to Microsoft Excel. The data were in column A, (time), B (x co-ordinate), C (y co-ordinate) and D (area). In column H the distance (pixels) between sequential points was calculated using simple geometry (as described in Chapter 2)

Formulas used :
Column F: *B6-B7*
Column G: *C6-C7*
Column H: *SQRT(POWER(F6,2)+POWER(G6,2))*



The directionality rule on X axis

What had made this rule a necessity was the problem of edge effect (described in 'Tracking aphids' section in Chapter 2). What was most characteristic about this problem was the continuous flipping of the centre of gravity between positions. This kind of movement when observed over such a time period will have very low directionality.

The movement of the insects is studied on one axis at a time on the attempt to define when an insect is mobile. The results of both axis analysed the same way and are combined at the end of the analysis.

Direction: when the insect is moving the result from formula (x_n - x_{n+1}) (*column F* in Figure-App. 1-3) can be:

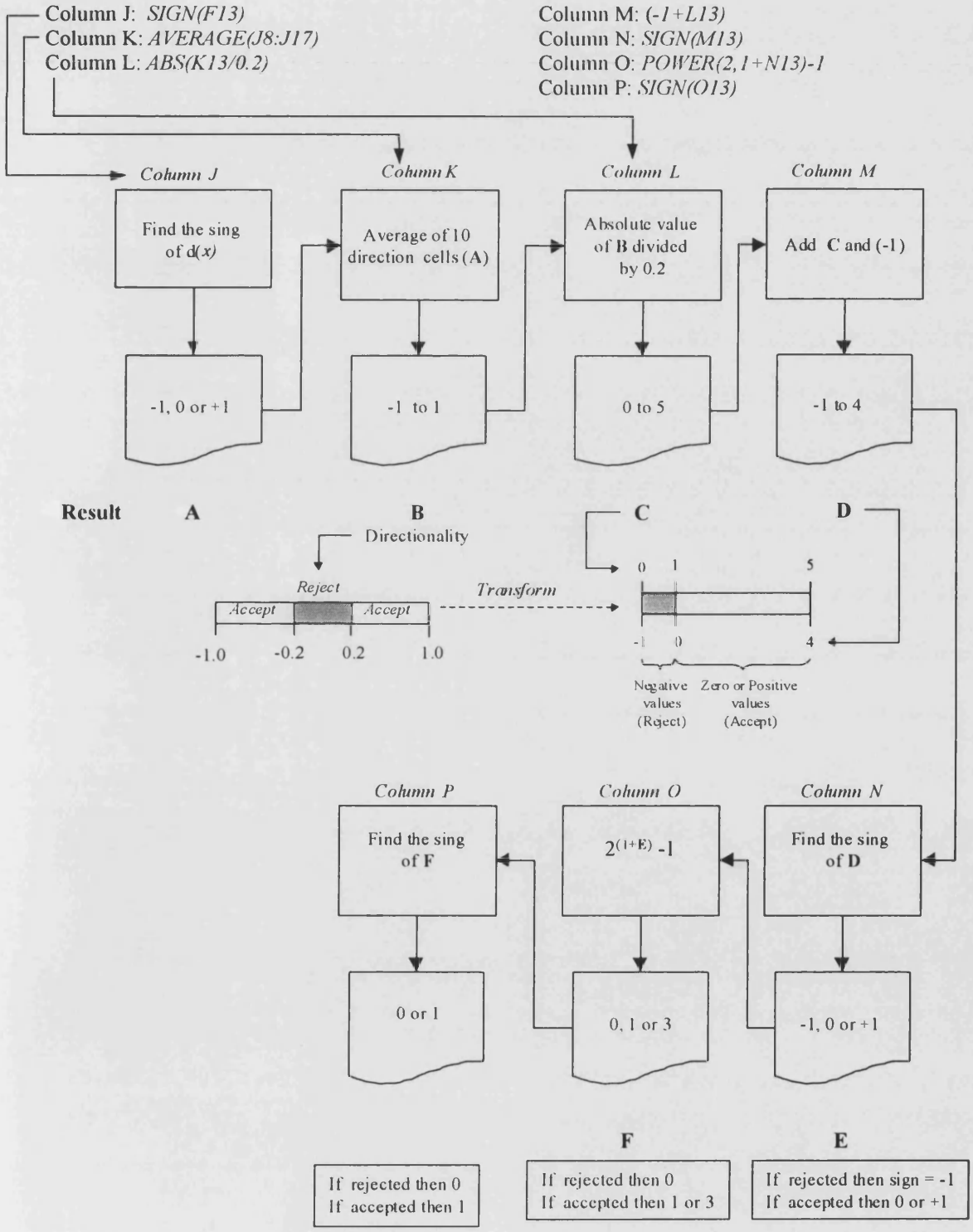
a positive number = translated as: the insect moving forward on the X axis (>>>)
a negative number = insect moving backwards on the X axis (<<<)
zero value = insect not moving or moving vertically to the X axis

The direction of the movement is characterised by the sing of the resulting number (+, - or 0).
The direction is calculated in column J by the formula: *SIGN(F6)*, which gives as an output the sing of the number (+1, -1 or 0).

Figure App-2.3 Directionality rule

The formula on Column J is producing result **A** (in this case the sign of the number d(x)). What the formula is doing is described in the square box and the result of the formula is in the 'result' box. The formula of the next column (K) is using this result (A) to produce a new result **B** (directionality)... ect. The formulas used by in Excel program are listed below:

Formulas used:



Directionality of the movement: The average direction of 10 cells.

Output = -1 to 1

Rule:

An insect is considerate to be mobile when there are at least 2 cells with the same direction more than the cells with the opposite direction within a range of 10 cells. The 10 cells tested are the current cell, the previous 3 cells (as history of the movement) and the next 6 cells (as future of the movement).

Table App-1-1. Examples of directional and non directional movement events

	Direction:	*-1*	*0*	*1*	*Total*	*Average (total/10)*	
Example 1	No of cells:	1	4	5	1×(-1) + 4×(0) + 5(1)=4	0.4	*Accept- Mobile*
Example 2	No of cells:	3	2	5	3×(-1) + 2×(0) + 5(1)=2	0.2	*Accept- Mobile*
Example 3	No of cells:	3	3	4	3×(-1) + 3×(0) + 4(1)=1	0.1	***Reject - not mobile***

Data from 10 cells (frames) describes a time period of about 10 to 15 seconds (depending on the time lapse mode), long enough to describe even a very slow mobility event.

The rule above when translated in numbers looks like this: the minimum acceptable directionality (average direction of the 10 cells (result A)) a is 2/10= 0.2 (if movement is forward, -0.2 if backwards) (as in Table App-1-1). By transforming this result (result B) to result C, the acceptable directionality is now equal or higher that 1on a scale of 0 -5. The following steps (results D - F) are designed so as to get an output of

0 if the directionality is rejected and

1 if it is acceptable

Combination of the both axis for final the rule result

An insect is considerate mobile if it the movement on at least one axis shows directionality.

Formula used: $y = \frac{a+b}{2^{(a+b)}} \times 2$,

Result: $y = 1$ if $(a = 1,\ b = 1)$ or $(a = 1,\ b = 0)$ or $(a = 0,\ b = 1)$

$y = 0$ if $(a = 0,\ b = 0)$

The selected distance

The distance that is covered when the insect is mobile is selected multiplying the distance array (Column H) with the result from directionality rule.

The real time

The time that the frame was recorded multiplied with the time lapse compression factor is giving the real time elapsed.

Appendix 3 - Insensitivity of aphids to red light

TABLE 6.5. THE SPECTRAL SENSITIVITY OF THE PHOTOPERIODIC RESPONSE

Species	Effective wave-lengths in nm	Non-effective wave-lengths in nm	References
1. Red-insensitive species			
Bombyx mori	350–510	>600	Kogure (1933)
Grapholitha molesta	430–580	<430; >600	Dickson (1949)
Panonychus ulmi	365–540	>600	Lees (1953a)
Dendrolimus pini	violet–green	red	Geispitz (1957)
Pieris brassicae	violet–green	red	Geispitz (1957)
	400–520	>580	Claret (1972)
Antheraea pernyi	398–508	>580	Williams *et al.* (1965)
	400–500		Hayes (1971)
Euscelis plebejus	365–550	>550	Müller (1964)
Anthonomus grandis	blue–orange	red	Harris *et al.* (1969)
Carpocapsa pomonella	400–500	>600	Norris *et al.* (1969)
	peak at 450		Hayes (1971)
Chaoborus americanus	peak at 550		Bradshaw (1969)
Megoura viciae	peak at 450–470	>550	Lees (1966, 1971)
2. Red-sensitive species			
Acronycta rumicis	407–655		Geispitz (1957)
Leptinotarsa decemlineata	423–675		De Wilde and Bonga (1958)
Pectinophora gossypiella	480–680		Pittendrigh *et al.* (1970)
Nasonia vitripennis	480–640	>650	Saunders (1975a)
Pimpla instigator	386–700		Claret (1973)

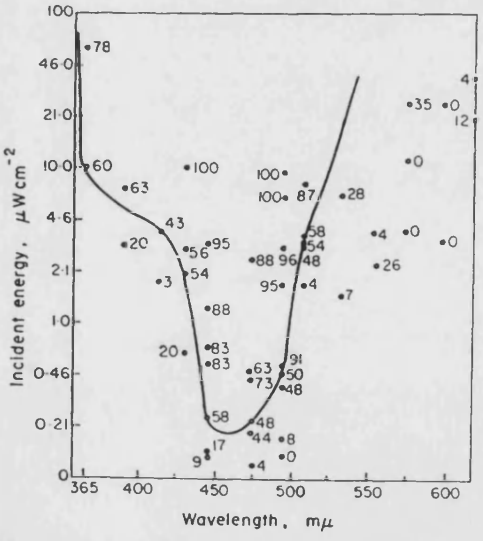


FIG. 6.14. Action spectrum for the photoperiodic control of progeny type in the aphid *Megoura viciae*. Semilogarithmic plot showing the response to 1-hour interruptions of monochromatic light introduced into a 10.5-hour dark period 1.5 hours after its inception. The "main" photoperiod of 13.5 hours was of white light (100 ft-candles). The intensity of the response is given by the percentage of aphids that produced viviparous progeny. These values are also shown by their respective points and the curve is drawn at approximately the 50 per cent response level. (From Lees, 1966.)

Table 6.5 and Figure 6.14 from Saunders D. S. **(1982)** *Insect clocks*, Pergamon Press, Oxford

Appendix 4 - Best line fit for the offspring birth rate experiment

The data were fitted to a simple linear regression following the method of least squares.

Aphid clone	*Linear Equasuion*	*Coefficient of determination R^2*
R1 aphid 1	y = 5.1x - 1.1	R2 = 0.9897
R1 aphid 2	y = 4.5x - 0.1	R2 = 0.9680
R1 aphid 3	y = 3.7x + 4.1	R2 = 0.9587
R1 aphid 4	y = 5.0x - 2.6	R2 = 0.9952
R1 aphid 5	y = 6.1x - 0.1	R2 = 0.9473
R1 aphid 6	y = 7.0x - 3.6	R2 = 0.9855
R1 aphid 7	y = 8.3x - 5.5	R2 = 0.9938
R2 aphid 1	y = 8.0x + 1.8	R2 = 0.9988
R2 aphid 2	y = 7.0x - 1.2	R2 = 0.9984
R2 aphid 3	y = 8.8x - 4.6	R2 = 0.9918
R2 aphid 4	y = 7.1x + 1.3	R2 = 0.9823
R2 aphid 5	y = 7.3x - 5.7	R2 = 0.9964
R2 aphid 6	y = 7.2x + 3.0	R2 = 0.9833
R2 aphid 7	y = 7.2x + 3.4	R2 = 0.9856
R3 aphid 1	y = 6.6x + 3.0	R2 = 0.9749
R3 aphid 2	y = 4.8x + 1.2	R2 = 0.9396
R3 aphid 3	y = 4.8x - 0.8	R2 = 0.9880
R3 aphid 4	y = 5.5x + 0.3	R2 = 0.9860
R3 aphid 5	y = 5.8x - 0.2	R2 = 0.9813
R3 aphid 6	y = 5.6x - 5.2	R2 = 0.9949
R3 aphid 7	y = 5.5x - 0.9	R2 = 0.9977